Environmental and Biotic influences on the Maintenance of Apomixis

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ABSTRACT

Most plant species that reproduce by the production of clonal seed (apomicts) retain some capacity for sexual reproduction (facultative apomixis). The frequency of this residual sexual reproduction in apomicts varies, even within a species, and this variation has often been attributed to the environment. This study aims to investigate the role of the environment on the expression of apomixis in the facultative apomict, *Hieracium pilosella*. *H. pilosella* has been chosen for this study because it is a widespread, exotic, species that possesses a facultative reproductive system. Additionally, there is interest in its' reproductive pathway as a potential source of genetic material for apomictic cropping technology.

Field experiments examining the reproductive mode of *Hieracium pilosella* found there was a positive relationship between the frequency of sexual reproduction, and both temperature and rainfall for the seven days prior to pollination (at capitulum anthesis). The frequency of sex in all experiments was determined by pollinating *H. pilosella* with a marker pollen donor, either the morphologically distinct *H. aurantiacum,* or transgenic accessions of *Hieracium* spp. with a kanamycin resistance insert, and using hybrid characteristics as a measure. Both field and glasshouse studies demonstrated a genetic component to the frequency of sex; while neither photoperiod, nutrient level, or the presence of a powdery mildew (*Erysiphe spp.*) had a significant effect on residual sexuality. Additionally, it was demonstrated that sexual events, although contributing only 0.2 – 6.0 % of total seed progeny in apomictic populations, are important in determining population genetic diversity.

The existence a flexible reproductive strategy indicates that both reproductive pathways are beneficial, or at least the cost of reproducing sexually (as predicted by evolutionary models) is insufficient to lead to its' loss. The maintenance of this rate of sexual reproduction, and the potential for it to generate population variation, may increase the complexity of both the transfer of genetic material from *Hieracium pilosella* to crop species, and biological control.

Chapter I. GENERAL INTRODUCTION

I.1.1 Scope and Rationale of the Study

Apomixis

Apomixis, or the production of clonal seed (agamospermy) in plants via parthenogenesis, has been described in a wide range of angiosperm taxa. The term apomixis has sometimes, mostly historically, been used to describe asexual reproduction in higher algae, mosses, ferns and animals; literally apo "away from" and mixis "act of mixing" in the widest sense (Asker & Jerling 1992). This study restricts the use of this term to seed production in higher plants, following the system of Nogler (1984), Mogie (1992) and Asker and Jerling (1992) [apomixis *sensu stricto*: agamospermy, seed apomixis]. Others, most notably Richards (1991) and Gustafsson (1946), include vegetative propagation as a form of apomixis. The reason for excluding this definition from the term apomixis will be discussed later (see Chapter I.2.9). Apomixis is contrasted by amphimixis, sexual reproduction involving the fusion of gametes to form a zygote [amphi "both", mixis "act of mixing"] (Asker & Jerling 1992).

Apomixis in *Hieracium* subgen. *Pilosella* is of the aposporous type (see Figure 1.2.2), first described in subgen. *Pilosella* by Rosenberg (1906), with the aposporous initial developing from the somatic cells of the nucellus, typically in the central region of this structure (see Figure 1.2.3). This form of apomixis is widely distributed among plant families, but is uncommon in the Asteraceae and is absent from *Hieracium* subgen. *Hieracium*, where apomixis is of the diplosporous type (where the megaspore mother cell gives rise to an unreduced megaspore through the avoidance of the reduction division of meiosis) (see Figure 1.2.2). Aposporous apomixis is almost always associated with polyploidy, a trait that is common in all gametophytic apomicts (Asker & Jerling 1992). Often several aposporous initials are formed, but only one develops into an aposporous embryo sac (Nogler 1984, Koltunow 1993, Koltunow *et al.* 1995). Although the majority of seed is formed via apomixis, there is some potential for the fertilisation of reduced or unreduced gametes to form progeny sexually, following failure of the apomixis initial to arise or develop (Skalińska 1971, Chapman & Bicknell 2000). Endosperm production in most aposporous apomicts is pseudogamous (requiring pollination of the polar nuclei to

form triploid endosperm) but endosperm production in *Hieracium* subgen. *Pilosella* is of the autonomous type, with no pollination required. Therefore, unlike most other aposporous apomicts, *Hieracium* subgen. *Pilosella* do not require pollination to form viable seed. The mechanism of apomixis and the potential for sexual reproduction in *Hieracium* subgen. *Pilosella* will be further discussed in chapters I.2.5 and I.2.6. It should be noted that what is commonly termed the seed in *H. pilosella* is in fact an achene, a type of fruit with a dry fruit wall surrounding the seed proper. This work will follow the convention of reproductive studies in the Asteraceae and use the term seed to prevent unnecessary complication.

This work aims to determine the level of residual sexual reproduction in the aposporous species *Hieracium pilosella* L. (1753) (Asteraceae), and also determine the role of environmental and biotic factors on the expression of this trait.

Practical Applications

The practical applications of this study are twofold. Firstly, this work compliments the Crop & Food Research Ltd. Apomixis group investigation into the genetics of apomixis in *Hieracium* subgen. *Pilosella.* Crop & Food Research Ltd. are using *Hieracium* subgen. *Pilosella* to investigate the potential isolation of a gene or genes for apomixis for transfer to crop species. The transfer of apomixis to crop species could potentially revolutionise production, making it possible to fix heterosis in crop lines, or to propagate from seed, crops that are normally grown from tubers such as potato (*Solanum tuberosum*). The economic and practical benefits of this will be further discussed in chapter I.3.1. *H. pilosella* has been chosen as a possible donor species for several reasons. The main benefits of using *Hieracium* subgen. *Pilosella* are that they are autonomous aposporous apomicts, not requiring pollination to form endosperm, and also that apospory is a trait with relatively simple inheritance (Bicknell 1994a). The short generation time (seed set within 3 to 4 months of germination) and ease of cultivating this plant under glasshouse conditions is also an advantage. These factors, combined with simple flower induction requiring only five or more days of "long day" conditions (Yeung 1989), make it an ideal species for such investigations. *H. pilosella* also has a relatively small genome, and is comparatively amenable to transformation. Both sexual and apomictic bio-types are available (Bicknell 1994a), and assessing apomixis is easily

achieved by decapitation of the immature capitulum (see Figure 1.2.1). Furthermore, *Hieracium* subgen. *Pilosella* has functional pollen formation to allow easy hybridisation to maternal sexual plants (Koltunow *et al.* 1995).

The second potential application of this work is to the bio-control programme for *Hieracium pilosella.* The success of bio-control programmes for various species have often been implicitly linked to the population structure of the target (see Barrett 1992, Cullen & Hasan 1988, Burdon & Marshall 1981). At present, the population and reproductive dynamics of this species are only partially understood, a neglected area being the potential for evolution due to residual sexual reproduction in apomictic plants. Previous ecological studies of *H. pilosella* in New Zealand have assumed a clonal reproductive mode (Jenkins 1995, Jenkins & Jong 1997), and the assumption that populations possess low variation (Makepeace 1981, Jenkins 1992). This work aims to quantify the extent and importance of sexual reproduction; to determine whether this is a source for the high levels of variation that have been detected in recent works (see Chapman *et al.* 2000 as *Pilosella officinarum,* Chapman & Brown 2001), and to gauge the potential for future evolution. The applications of breeding system to bio-control have become unclear in *H. pilosella* due to current controls having wider specificity. Consequences for bio-control of *H. pilosella* will be discussed in chapter I.3.2.

History of *Hieracium pilosella* in New Zealand

Hieracium pilosella was first introduced into New Zealand from Europe in the 1860s, as a contaminant of pasture seed. The origin of *H. pilosella* in New Zealand is unknown, but it is often suggested Scandinavia is the most likely source, due to the large exports of pasture seed from this area in the late 1860s (D. Scott *pers comm.*). *H. pilosella* is characterised by its single pale yellow capitulum inflorescence and stoloniferous growth. Its' common name of "mouse-eared hawkweed" refers to the small, hairy, curved leaves. This prostrate, clonal, species has displaced a large proportion of the native tussock grasslands in the New Zealand high country, and also prevents the establishment of pasture species on farmland (Jenkins 1992). The "ruderal" (*sensu* Grime 1979) nature of *H. pilosella* makes it an efficient invader of bare ground and existing vegetation stands, particularly on deficient soils (Makepeace *et al.* 1985, Jenkins 1992, Scott 1993a, 1993b).

Others have suggested ecosystem degradation as the key to the success of *H. pilosella* (Treskonova 1991, Johnstone *et al.* 1999). This will be further discussed in chapter I.2.2.

This study aims to determine if reproductive patterns in New Zealand *Hieracium pilosella* populations are related to environmental conditions, and if it is therefore possible to use these as a predictor for areas with higher than normal levels of sex. The identification of environmental conditions that encourage asexual reproduction will have consequences for the application of genetic material from this species to apomixis gene programmes, and may allow more accurate targeting of specific bio-control agents.

I.1.2 Environmental control of Apomixis

The proportion of progeny of sexual and apomictic origin in facultative apomicts has been shown to vary over environmental gradients, particularly in the Gramineae. Environmental cues for the expression of apomixis have been investigated, mostly over natural environmental or latitudinal gradients. These studies aimed to determine the proximate cue for the differential expression of apomixis thought to occur over such gradients.

Early studies included an investigation of the environmental conditions that controlled apomixis in *Dichanthium aristatum* (Gramineae) (Knox & Heslop-Harrison 1963, Knox 1967). Temperature, rainfall, and photoperiod were examined over a latitudinal gradient of 27 degrees. Two strains of *D. aristatum* were used, and the amount of apomictic reproduction determined by cytological methods. It was found that whilst a decrease in rainfall and temperature did correlate well with an increase in apomixis, photoperiod had the best relationship with apomictic reproduction, due to the more predictable nature of this variable. As day-length decreased, the proportion of apomictic to sexually produced seed increased. Day-length was also found to be a good predictor of apomixis in the grass *Calamagrostis purpurea* and in *D. annulatum,* again with the proportion of seed produced via apomixis decreasing as day-length increased (Nygren 1951, Brooks 1958, In: Knox 1967). All of these plants are aposporous apomictic members of the Gramineae.

Evans and Knox (1969) found a similar pattern for the control of apospory in *Themeda australis* (kangaroo grass). As photoperiod decreased, the amount of seed produced by apospory increased, as in the previous studies (Knox & Heslop-Harrison 1963, Knox 1967). This study is unusual in that the effect was found for the pre-treatment of the plants, before exposure to the various experimental photoperiods had begun. The statistical significance of this finding was difficult to interpret with the authors violating the assumptions of the Chi-square test they applied. The pooled Chi-square test should only be applied if the null hypothesis is not rejected in any of the cases. Evans & Knox (1969) state the null hypothesis is already rejected for two of the six races examined, violating the assumption of the heterogeneity Chi-square analysis (pooled Chi-square) (Zar 1996). Therefore it is probably more correct to say that Evans & Knox (1969) found an effect of photoperiod for two of the twenty races of *T. australis* in their experiment.

Other studies have shown no effect of photoperiod on the expression of apomixis. McWilliam *et al.* (1970) found no significant effect of photoperiod on the proportion of seed produced by apospory in four of five races of *Hyparrhenia hirta* and concluded that photoperiod had no effect on the degree of apomixis. Burton (1982) also found no effect of environment on the breeding system of *Paspalum notalum* (bahiagrass). Hussey *et al.* (1991) found that two different accessions of buffelgrass (*Pennisetum ciliare* (L.) Link [= *Cenchrus ciliaris* L.]) did not display any difference in reproductive mode under 8, 12 & 16 hour treatments. This was also found to be the case in a three year field study, where the same photoperiods in spring and autumn were associated with maximum and minimum frequencies of sexual embryo sacs respectively (Hussey *et al.* 1991).

A further study of buffelgrass found that the presence of inorganic salts altered the embryo sac development in both apomictic and sexual plants (Gounaris *et al.* 1991). The addition of inorganic salts was concluded to support the formation of Polygonum type embryo sacs (the sexual eight nucleate embryo sac that is the most common type in angiosperms) in aposporous apomictic individuals. It was also found that in sexual accessions the addition of salts to the soil induced multiple embryo sacs. It was concluded that it was most likely water stress at the cellular level was influencing cell growth and differentiation, and ultimately the embryology (Gounaris *et al.* 1991).

All of these studies indicate that there is an environmental component to apomixis, but it is currently unclear as to whether cues with any universality exist.

I.1.3 Geographic distribution of Apomixis – the "General Purpose" Genotype

Native European populations of *Hieracium pilosella* are often comprised of both sexual and apomictic individuals, and there is evidence that the proportion of each varies considerably over geographic and environmental gradients. For example, apomictic biotypes are most often restricted to higher altitude / latitudes than sexuals, this phenomenon often termed "geographic parthenogenesis" (Gadella 1972, 1991a, 1991b). Although this pattern has been observed in many different taxa (see Asker & Jerling 1992 for a review), the reasons for this are not well understood. One of the most convincing explanations to date has been proposed by Bierzychudek (1989), and has been termed the "general-purpose" genotype model (see also Lynch 1984). This model is relevant to polyploid plant taxa, as almost all plants with the ability to set seed via apomixis are (Mogie 1992). Bierzychudek (1989) grew populations of *Antennaria parvifolia,* consisting of either obligate sexual or apomictic individuals, in a common garden situation. She found that apomicts had a competitive advantage over sexual individuals under all the environmental treatments examined. Apomictic individuals of *A. parvifolia* in the field are restricted to higher latitudes and altitudes than amphimictic plants, as is also the case in *Hieracium* subgen. *Pilosella* spp. (see Gadella 1987). Under glasshouse conditions it was found that the apomictic individuals had higher flower head output, greater biomass production (although not significantly so), and greater survival in extreme environments. The coefficient of variation for biomass production was also significantly lower in apomictic plants. This demonstrated that apomicts were far more resistant to extremes in conditions, but it was also found that they out-competed amphimicts in the more favourable conditions from which they are usually excluded. It was concluded that the apomicts represent a "general purpose genotype"; one which could withstand a greater range of environmental conditions than the "specialised" amphimicts. The apomicts displayed much lower "environmental sensitivity", and therefore may have reduced chances of extinction if faced with rapid environmental changes (Bierzychudek 1989).

The origin of apomictic taxa, postulated to be hybridisation between sexual individuals followed by polyploidy, was suggested as a potential explanation of these findings. The resulting genotypes have high levels of internal heterozygosity, and particularly in apomictic taxa of the Asteraceae, extreme levels of plasticity. The remaining question is if apomicts under experimental conditions were most superior in hot, dry environments, why are they restricted to cooler, wetter, high altitude / latitude areas under natural conditions? (Bierzychudek 1989).

Bierzychudek (1989) states that when comparing sexual and asexual reproduction within taxa, it is necessary to discard the assumption that the only difference between the two reproductive types is that sexual individuals can produce genetically diverse progeny. The difference in environmental tolerance that *Antennaria parvifolia* exhibits is clear evidence for this.

A similar finding was reported by Michaels and Bazzaz (1989), who found that apomictic individuals of *Antennaria parlinii* had greater plasticity in biomass response to nutrient and light gradients, particularly in reproductive structures, when compared to sexual plants. Again, there was strong niche differentiation between the two types, although in this case it was suggested that apomictic *A. parlinii* seed and ramets have lower survival rates than sexuals.

Antennaria parvifolia and *A. parlinii* are unusual compared to most taxa with both sexual and apomictic individuals in that both types are highly polyploid (*A. parvifolia* - octoploid to decaploid, *A. parlinii*–hexaploid). In most other cases, the sexual is diploid, or perhaps tetraploid, whilst apomicts commonly have much higher ploidy (Asker & Jerling 1992).

An Application of the "General Purpose" Genotype

The application of such a model may be more appropriate to a species such as *Hieracium pilosella.* The difference between the two species is that unlike *Antennaria parvifolia, H. pilosella* has different ploidy levels associated with different reproductive modes. In Europe, where *H. pilosella* is native, sexuals are often diploid or tetraploid

where they naturally occur, with apomicts being most often pentaploid, or rarely hexaploid or septaploid (see Chapter I.2.3). Gadella (1991b) provides a report of sexual hexaploid *H. pilosella* in the French, Italian and Swiss Alps, but these have a restricted distribution compared to tetraploid and pentaploid cytotypes (Krahulcová & Krahulec 1999). In New Zealand, sexuals are thought to be absent (Jenkins 1995 – although see Chapman *et al. in prep*), and large proportions of individuals have high (4x to 7x) ploidy levels (Chapman & Lambie 2000 as *Pilosella officinarum*). In general, an apomict will have a higher ploidy level than a sexual, and this is especially true of the higher (6x to 7x) ploidy levels in *Hieracium* subgen. *Pilosella*. As apomicts have more copies of alleles at a single locus, they theoretically have a greater potential for heterozygosity, which may confer an increase in environmental tolerance. This theory relies on environmental variation limiting the range of sexual individuals, and we would therefore expect apomicts to be more common in areas with extreme fluctuations in physical conditions, with sexuals restricted to relatively "uniform" or "favourable" areas. This has even been observed in sexual taxa with both diploid and tetraploid individuals (*Capsella rubella* and *C. bursa-pastoris* respectively), with tetraploids having "greater genetic flexibility" than diploids (Neuffer & Eschner 1995).

Work by Peck *et al.* (1998) has suggested that the key factor for "geographic parthenogenesis" is length of growing season. They suggested that the lower fecundity of all plants at high latitude would favour apomixis if this was also combined with a fitness loss in sexuals due to these plants being better adapted to a longer growing season, due to their more southerly distribution. Although attractive, this theory doesn't consider more subtle genetic differences between apomicts and sexuals of the same taxa, something that Bierzychudek (1989) and Michaels and Bazzaz (1989) emphasised in their work.

I.2 *Hieracium pilosella*: a Model for the Isolation of Apomixis.

I.2.1 Taxonomy

Taxonomy follows that used in the adventive Flora of New Zealand (Webb *et al.* 1988). These authors retain the older classification for *Hieracium* spp., following the system of Linnaeus in the Species Plantarum of 1753, and retaining the single generic rank. Two subgenera are found in New Zealand, subgenus *Hieracium,* and subgenus *Pilosella.*

Subgenus *Pilosella*

In New Zealand four species and one hybrid are described in the subgenus *Pilosella,* and it is these that this study concentrates on. The species are: *Hieracium pilosella* L., *Sp. Pl.* 801 (1753), *H. aurantiacum* L., *Sp. Pl.* 801 (1753), *H. praealtum* Gochnat., *Tent. Pl. Cich.* 17 (1808) and *H. caespitosum* Dumort., *Fl. Belg.* 62 (1827). The hybrid is *H.* x*stoloniflorum* Waldst. et Kit., *Pl. Rar. Hung. 3*:303 (1812), an apomictic hybrid between *H. pilosella* and *H. aurantiacum.* This subgenus is characterised by stoloniferous growth, is often sexual or partly apomictic and has pappus hairs in one row (Webb *et al.* 1988).

Subgenus *Hieracium*

Five species are described in the subgenus *Hieracium* in New Zealand. In contrast to subgenus *Pilosella*, members of the subgenus *Hieracium* lack stolons are almost always obligate apomicts, and the pappus hairs are partly in two rows. The five species are *H. argillaceum* group, *H. lepidulum* (Stenstroem) Omang, *Nyt Mag. Naturvid. (Christiania) 43*: 291 (1905), *H. murorum* L., *Sp. Pl.* 801 (1753), *H. pollichiae* Shultz-Bip., *Pollichia 13*: 23 (1855), and *H. sabaudum* L., *Sp. Pl.* 801 (1753). The species of this subgenus are not as common or widespread as those in subgenus *Pilosella* in New Zealand.

The Flora of the British Isles follows a different classification system, with subgenus *Pilosella* elevated to generic rank (Stace 1997). The species comprising this genus are then referred to as *Pilosella officinarum* (L.) F.W. Schultz & Sch. Bip. (*Hieracium pilosella*), *P. aurantiaca* (L.)F.W. Schultz & Sch. Bip (*H. aurantiacum*), *P. praealta* (L.)F.W. Schultz & Sch. Bip (*H. praealtum*), *P. caespitosa* (L.)F.W. Schultz & Sch. Bip. (*H. caespitosum* and *P.* x*stoloniflora* (Waldst. & Kit.)F.W. Schultz & Sch. Bip. (*H.*

x*stoloniflorum*). All species names from here on will follow Webb *et al.* (1988) as is the convention in New Zealand botany, unless stated otherwise.

Subspecies in the subgenus *Pilosella*

The rank of subspecies will be ignored in this work for two reasons. Firstly the lack of correlation between the New Zealand Flora descriptions and field material (Chapman & Brown 2001) makes assigning subspecies difficult. Secondly, the relevance of this taxonomic rank is unclear in a study such as this, examining population and individual differences in reproductive mode under varying conditions. The Flora of New Zealand Volume IV states, nevertheless, that *Hieracium aurantiacum* in New Zealand is subspecies *carpathicola,* and *H. pilosella* is most often subspecies *micradenium,* but occasionally subspecies *pilosella* [recorded in Hawkes Bay, Canterbury, Otago and Southland] or *trichosoma* [Ruahine Range, Canterbury and Otago]. *H. praealtum* is subspecies *bauhinii,* but is sometimes described as subspecies *praealtum. H. caespitosum* is not described to the subspecies level (Webb *et. al* 1988).

It should also be noted that *Hieracium praealtum* Gochnat., *Tent. Pl. Cich.* 17 (1808), is often cited as *H. bauhinii* Besser., *Prim. Fl. Galic* 2 (1809) in Europe (S. Bräutigam *pers comm.*), but for the purposes of this work *H. praealtum* will be adopted. Additionally, records of *H. pratense* and *H. piloselliodes* in New Zealand refer to *H. caespitosum* Dumort., *Fl. Belg.* 62 (1827) (Webb *et al.* 1988).

I.2.2 Economic and Environmental Impacts of *Hieracium* spp.

Hieracium pilosella, or mouse-eared hawkweed, is a major economic and environmental weed in New Zealand. *H. pilosella* and the other *Hieracium* subgen. *Pilosella* spp., have been present in the New Zealand high country since at least the 1860s, after entering the country as contaminants in pasture seed (Makepeace 1985b). In the last 30 to 40 years the range of *H. pilosella* has dramatically increased to its current distribution (Johnstone *et al.* 1999), resulting in devastation of pastoral areas and indigenous tussock grasslands (Scott 1993a, Rose *et al.* 1995, Johnstone *et al.* 1999).

Hieracium pilosella was first reported as a potential weed in New Zealand in 1864 by W.T.L. Travers (in Lambie 1999) who clearly saw the potential problem of this species, but this was not widely appreciated until the late 1970s (Treskonova 1991). Connor (1992) reports the results of a survey of the extent of *Hieracium* spp. in Canterbury during the 1960s. Although the author acknowledged the widespread nature of the species, at the time of the survey the potential invasiveness went unrecognised (see Connor 1964). Moore (1955) saw the potential for spread of *Hieracium* spp. in tussock grasslands, but ranked it behind white clover and sheep's sorrel for invasive potential. Allan in 1924 emphatically stated the need for control of *H. pilosella.* Of *H. pilosella* in Canterbury he said "This is a case where timely action will prevent a great deal of subsequent bother" (Allan 1924). Unfortunately, this warning was not heeded at the time.

Distribution and Extent

Hunter (1991) categorised the distribution of several *Hieracium* spp. in the South Island based on observations and surveys by a variety of parties, ranging from scientists to run-holders. Four categories of infestation were described, depending on the extent of the invasion. 'Present' referred to areas where *Hieracium* spp. on average comprised 1% or less of total ground cover, 'common' was 1 to 20% of total ground cover, 'conspicuous' 20-50%, and 'dominant' greater than 50%. Over 500 000 hectares of land was identified as being in the 'dominant' category, 1 000 000 hectares in the 'conspicuous' category, while these made up less than half of the actual land area with *Hieracium* spp. present in the South Island (Hunter 1991). This represented a potential risk to one million sheep-equivalent stock-units and at 1994-5 export prices a potential financial loss to high country production of $78.1 million dollars. Since the time of this study the distribution of *Hieracium* spp. has both widened and intensified (J. Aspinall *pers comm.*).

Scott (1992) showed that populations of *Hieracium* spp. in the Waimakariri basin had exponentially increased their ground coverage in a 35 year period. Scott (1993a) reported the 1957 average cover of *Hieracium* spp. over seven sites was 0.3% of total ground cover, with the highest site density 1.2%. In 1993 when the study was completed the average percentage ground cover of *Hieracium* spp. was 6.5%, with a maximum value of 15.8%; on average a 23-fold increase in the 35 years of the study (Scott 1993a). This

study overlaps the field-sites of the present work, and it is clear *Hieracium* spp. are still increasing their range in this area (G.J. Houliston *pers obs.*).

Johnstone *et al.* (1999) examined 66 sites in Eastern Otago and showed that between 1982 and 1992, *Hieracium pilosella* populations had increased in cover at 61 of the sites. Of the five sites where it did not increase, it had comprised less than 1% of the total cover in 1982, and was absent when the sites were re-examined. The reason for this was thought to be a programme of land improvement for grazing purposes, particularly the addition of granular fertilisers. Of the sites that had increased in *H. pilosella* cover, the mean rate of increase was 4% per year. The amount of bare ground at these sites had also increased, but this was attributed to *H. pilosella* increasing more rapidly at sites with greater degradation (Johnstone *et al.*1999).

Reasons for the success of *Hieracium pilosella*

The high country pasture land that *Hieracium pilosella* has been so successful on is characterised by yellow-brown soils which are nitrogen, phosphorous, sulphur and molybdenum deficient (Johnstone *et al* .1999). It has been suggested that *H. pilosella* can depress levels of nitrogen in the soil, giving it a competitive advantage over pasture grasses with high nitrogen requirements (Makepeace 1985a). Boswell and Espie (1998) showed that *H. pilosella* mats tied up the available nutrients and water in the area surrounding them, creating a 'halo' of poor quality habitat. If nutrient levels are high, the resulting halo is not as severe, and it will be possible for other species to survive adjacent to *H. pilosella.* As nutrient levels fall, *H. pilosella* becomes more dominant as it displaces its' neighbours by creating poor quality substrate (Makepeace *et al.*1985, Boswell & Espie 1998). Others have suggested the 'halo effect' of *H. pilosella* is due to allelopathic effects, with *H. pilosella* degrading the soil by the production of umbelliferone in the leaves, and the subsequent release of this compound with senescence (Makepeace *et al.* 1985). This has been largely disputed by later works, particularly Henn *et al.* (1988) and Fan and Harris (1996). Davis (1997) compared the nutrient responses of *Pinus radiata, Trifolium repens, Dactylis glomerata* and *H. pilosella* under different nutrient regimes and found that the response of *H. pilosella* to increased nitrogen and sulphur was greatest of the four species examined, indicating the high plasticity of this species.

Changes in the economic policies of New Zealand in the early 1980s resulted in the removal of subsidies to high country farmers for land improvement. The subsequent decrease in fertiliser use has been suggested as being responsible for the increase in *Hieracium pilosella* densities at this time (Jenkins 1992). Fan and Harris (1996) suggest a more complex situation in respect to nutrient regimes in the high country. They suggest the increase in fertiliser use in high country areas between the 1960s and 1980s resulted in an increase in nutrient levels in tussock grassland areas that had not previously been fertilised. These areas had already had the sward opened by the presence of stock and rabbits, and the subsequent low level fertilisation promoted the establishment of *H. pilosella.* Fan and Harris (1996) suggest that the rapid ability of *H. pilosella* to respond to nutrient levels conferred a competitive advantage over native tussock species and inter-tussock vegetation. Due to the findings of their of fertiliser trials, they suggest the low levels of fertiliser input encouraged both stolon and flower production, and facilitated the increase in population size observed during this period.

A contrasting view on this has been provided by Treskonova (1991). A comparison of vegetation structures of tall-tussock grassland between 1964 and 1989 concluded that invasion of grassland by *Hieracium pilosella* relied on the prior degradation of the sward. Causes of degradation were assigned to the impact of pastoral practices, particularly in the absence of land improvement, modification of the existing vegetation, particularly the removal of *Chionochloa spp.,* and an increase in weeds and bare ground (Treskonova 1991). This is contrasted by Rose and Frampton (1999) who found that *H. pilosella* establishment was independent on the availability of bare ground, although this study agreed with Treskonova (1991) on low vegetation being more amenable to invasion. It should be noted that Treskonova (1991) is comparing *H. pilosella* invasion into modified versus almost completely unmodified vegetation, whereas most other studies are based in grazed tussock grassland or pastoral situations.

The idea that *Hieracium pilosella* first depresses nutrient levels or relies on degradation of the sward to allow invasion has been rejected by a study of the patterns of *Hieracium* spp. density increase. As the increase in the cover of *Hieracium* spp. was found to be

exponential, it is predicted it is more akin to a species invading an available habitat, than one which has to first depress nutrient levels to facilitate it's establishment (Scott 1993a).

Other work has suggested that fire has played an important role in allowing the invasion of *Hieracium pilosella* into tussock grassland areas. For example, a population of *H. pilosella* established on the Lammermoor Range in central Otago following the burning of the existing tussock vegetation. The removal of inter-tussock litter and tussock biomass was seen as crucial to the invasive potential of *H. pilosella* (Allen *et al.* 1992). Duggan (1992) recorded the invasion of *H. pilosella* into an area of Glenshee station in Otago, following burning. A survey by Wilson (1992) after an accidental fire on the Liebeg range in Mount Cook National Park showed that *H. pilosella* comprised only 1% of the total vegetation cover, although the total range of the species had increased. Many studies agree that the combination of burning and grazing by rabbits results in opening of the existing sward, creating "safe sites" for *H. pilosella* invasion (Treskonova 1991, Allen *et al.* 1992, Jenkins 1992, Scott 1992, 1993a).

Recent work has concluded that neither the "aggressive invader" or "depleted ecosystem" hypothesis is sufficient to explain the invasion of *Hieracium pilosella* into the New Zealand high country alone, and that single factor approaches to the problem are also unlikely to be successful (Rose *et al.* 1995).

I.2.3 Ploidy Level

Studies of ploidy level in New Zealand populations of *Hieracium pilosella* have discovered numerous different ploidy states, including aneuploids (those with a ploidy level not an integer multiple of the base number) (Chapman & Lambie 2000). However, Makepeace (1981) reported only pentaploids (2n = 5x = 45) in a study of 31 populations from the South Island. Jenkins and Jong (1997) reported pentaploids and hexaploids (2n = 6x = 54) in a study of 34 locations in New Zealand, although hexaploids were restricted to one site (Haldon station, Mackenzie basin). Later works have confirmed that the most common ploidy level in New Zealand is 2n = 5x = 45, although tetraploids (2n = 4x = 36) (Chapman & Lambie 2000 as *Pilosella officinarum*, Houliston & Chapman 2001)

and septaploids (2n = 7x = 63) have also been reported (Chapman & Lambie 2000). This pattern is not uncommon in apomictic complexes such as *H. pilosella,* and it seems earlier works on this topic have underestimated ploidy variation in this species in New Zealand.

Ploidy variation in European populations of *Hieracium pilosella* is considerable, but differs over geographic ranges. German records of *H. pilosella* most frequently refer to tetraploids, and although 2x, 3x, 4x, 5x, 6x & 7x plants have been described, flow cytometric analysis of a location in Saxonia reported only tetraploid plants (Bräutigam & Bräutigam 1996). Other recent works have confirmed the tetraploid nature of German populations, although pentaploids and hexaploids are reported from Austria (Schuhwerk & Lippert 1997). Turresson & Turresson (1960) in a review of cytotypes from Scandinavia, state amphimictic tetraploid and apo-amphimictic pentaploids, hexaploids and septaploids are present. Gadella (1974, 1987, 1991a) states that Dutch populations are comprised of sexual tetraploids, and apomictic pentaploids and hexaploids. Czech records for *H. pilosella* are for sexual tetraploid plants, although some pentaploid apomictic accessions that resemble *H. pilosella* have been found at a single location (Krahulcová & Krahulec 1999, Krahulcová *et al.* 2001). Populations in Great Britain have been reported as diploid, triploid, and tetraploid in southern England, while pentaploids are restricted to Scotland (Bishop & Davy 1994). Whether triploid cytotypes in Great Britain are *H. pilosella sensu stricto*, or of interspecific hybrid origin, is something that is often questioned by taxonomists in central Europe (F. Krahulec *pers comm.*).

I.2.4 Ploidy Level and Apomixis

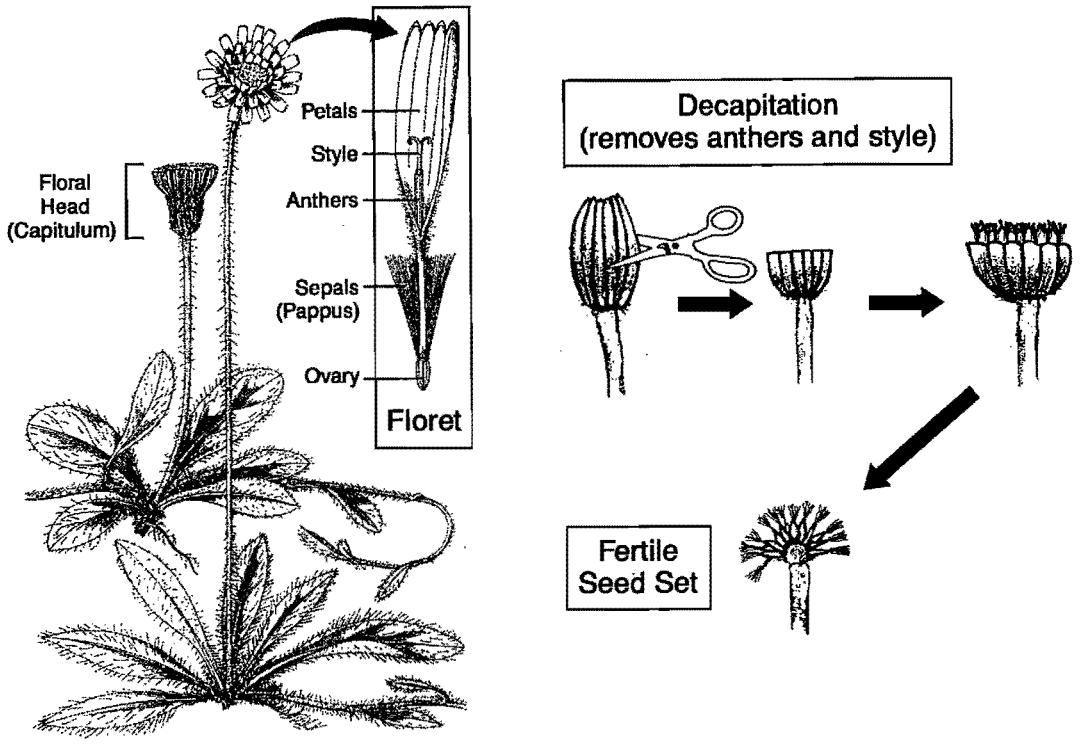
Ploidy level has been suggested as a correlate of reproductive mode in European field populations of *Hieracium pilosella*. Gadella (1972) examined the ploidy levels of different populations of *H. pilosella* and attempted to draw a correlation between ploidy level and breeding system. This work relied on cytological methods to determine the mode of reproduction, combined with an emasculation experiment (see Figure 1.2.1). Gadella (1972) claimed that tetraploid individuals of *H. pilosella* were obligate sexuals

due to the absence of filled seed following emasculation. Pentaploids would form seed if emasculated, but the amount of seed did vary.

Gadella (1987) states that in the populations in Europe that he examined, pentaploids were obligate apomicts, whereas tetraploids were almost entirely sexual with a few rare occurrences of facultative apomicts (apo-amphimicts). This conclusion was drawn because all of the seed progeny examined from pentaploids following pollination with a tetraploid was also pentaploid. Later works by the same author (Gadella 1991a, 1991b) have reiterated these findings, and this seems to be a widely accepted generalisation in Europe (see Stace 1997).

Early New Zealand literature on ploidy level in *Hieracium pilosella* reported only pentaploids and hexaploids (Makepeace 1985b, Jenkins 1992), although later works have identified tetraploid individuals (Chapman *et al.* 2000 as *Pilosella officinarum*, Houliston & Chapman 2001). As all *H. pilosella* described in New Zealand to date is primarily apomictic, the predictions of Gadella (1987) do not seem to apply, as New Zealand tetraploids, like pentaploids, are predominantly apomictic.

Figure I.2.1 The Emasculation test for Apomixis in *Hieracium*.



The removal of the top 5 mm of the capitula removes the style and anthers (see inset – floret diagram). If fertile seed is produced, this confirms that there is at least some capacity for apomixis (modified from Koltunow *et al.* 1995).

I.2.5 Reproduction in *Hieracium* subgen. *Pilosella*.

Hieracium pilosella possesses a complex reproductive system, which in part is probably responsible for its success as a pernicious weed in New Zealand (see Chapman & Brown 2001). As an invader of existing swards it is clear that the production of stolons gives it a competitive advantage over other species (Lamoureaux 1998), but until recently the importance of seed production has been ignored (see Makepeace 1985b). *H. pilosella* population structure in New Zealand is often complex, with far more variation present than would be expected for an obligate apomict reproducing primarily via stolons. Genetic and morphological studies have identified very few clones in populations, and clones present in more than one population are uncommon (Chapman *et al.* 2000 as *Pilosella officinarum*, Chapman & Brown 2001). The most likely source of such variation is from the production and establishment of seed resulting from amphimixis.

While the majority of seed produced by *Hieracium pilosella* in New Zealand is via apomixis (Webb *et al.* 1988, Jenkins 1992), there is potential for sexual reproduction (Chapman & Bicknell 2000) which may play a role in determining the genetic structure of *H. pilosella* populations. Viable seed in *H. pilosella* can be produced with or without both fertilisation and meiosis, and a full range of contingencies is possible. Embryology in *Hieracium* subgen. *Pilosella* can be seen in Figure 1.2.2. It should be noted that the sexual pathway as illustrated for tetraploid sexual *H. pilosella* can also occur in predominantly apomictic plants.

Mechanism of apomixis in *Hieracium pilosella*

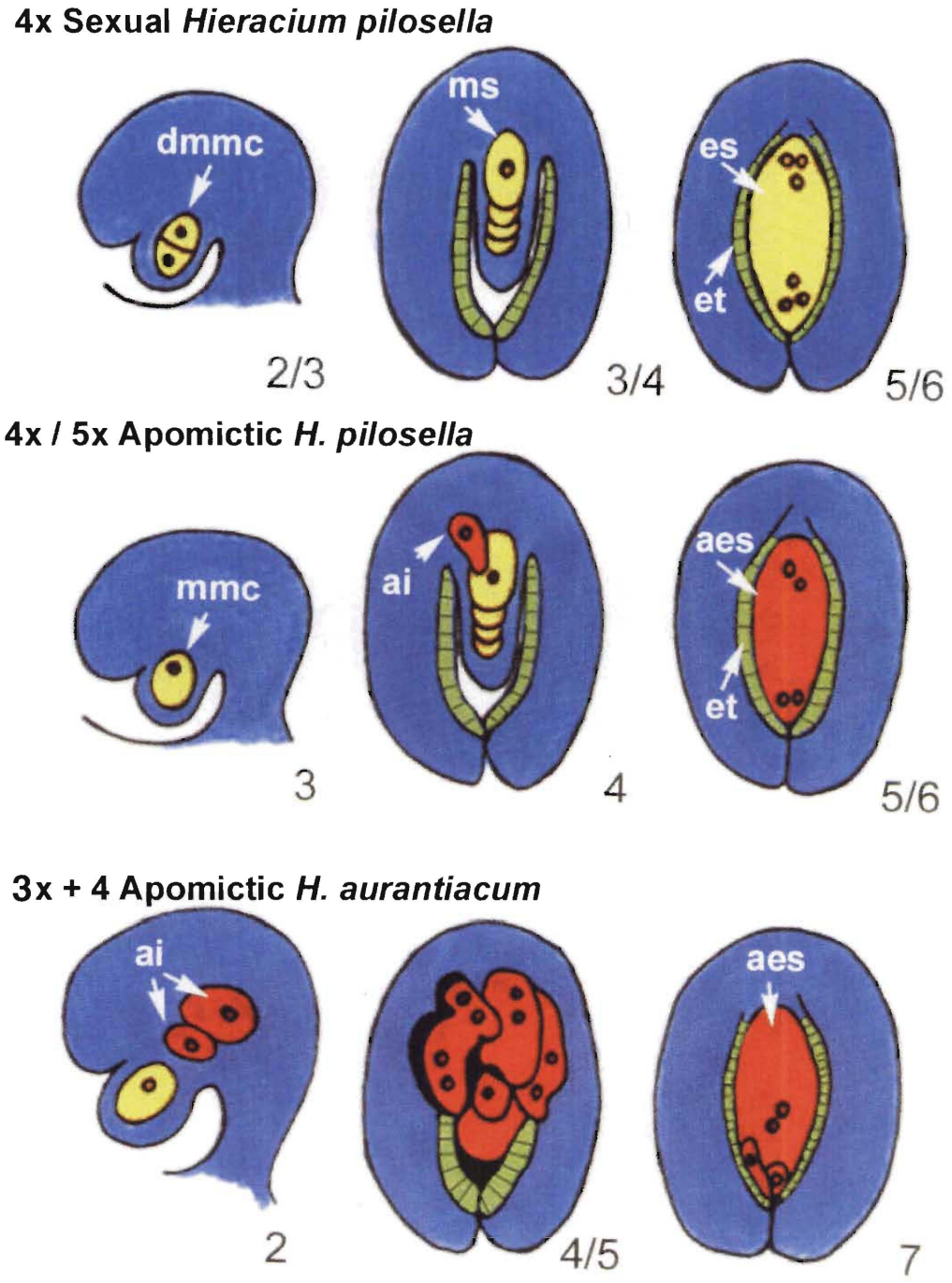
Seed produced without fertilisation in *Hieracium pilosella* is via aposporous apomixis (*sensu* Stebbins 1950). Unlike many other aposporous taxa, *H. pilosella* is autonomous, requiring no pollination for endosperm formation. Apomictic seed production in *H. pilosella* is via the production of unreduced embryo sacs, which arise directly from the somatic cells of the nucellus (apospory). The resulting eight nucleate embryo sac, after three mitoses, is genetically identical to the maternal type. This is often referred to as the "*Hieracium* type" (Asker & Jerling 1992) (see Figure 1.2.3). This does not preclude sexual reproduction, but the unreduced embryo sacs typically develop faster than their

meiotic counterparts and usually physically displace them, possibly due to the time it takes to complete meiosis. However it is possible for meiotic and aposporous embryos to coexist within the individual, or even within the ovule (polyembryony) (Koltunow *et al.* 1998). It is also possible for a reduced gamete to undergo mitotic divisions to form a viable embryo, with a reduced ploidy level (Asker & Jerling 1992). These polyhaploids can have varying ploidy levels, as meiosis in *H. pilosella* is often irregular. These are typified by low vigour and only arise rarely, and thus are not considered to be important in the short-term evolution of field populations (Bicknell & Borst 1996).

As is common to almost all apomictic taxa studied in detail, apomixis in *Hieracium pilosella* is facultative, and sexual reproduction is a periodic event (Chapman & Bicknell 2000). The amount of seed that is produced sexually in the field by *H. pilosella* is unknown, and will be addressed by this study. The production of seed sexually in *H. pilosella* can, like apomixis, take two different pathways. Again, these depend on whether meiosis occurs or not. The BII hybrid is formed when a reduced maternal megaspore is fused with a reduced microspore, and is the "normal" mechanism in sexual organisms. BIII hybrids (addition hybrids) also occur in *H. pilosella*, where an unreduced gamete is fused with either a reduced or unreduced other, resulting in a summation of the ploidy levels (Gadella 1991a).

All of these different reproductive pathways can result in viable offspring in *H. pilosella*, although little is known of their significance in natural populations. *H. pilosella* is self incompatible, with a sporophytic incompatibility mechanism (Asker & Jerling 1992, Krahulcová *et al.* 2000).

Figure 1.2.2 Sexual and Apomictic Embryology in *Hieracium* subgen. *Pilosella*.



The numbers at the base of each diagram refer to the stage of development of the capitulum (see Koltunow *et al.* 1998). Yellow indicates cells involved in sexual reproduction, red in apomixis. dmmc – dividing megaspore mother cell; ms – megaspore; es – embryo sac; et – endothelium; mmc – megaspore mother cell; ai – aposporous initial; aes – aposporous embryo sac (Modified from Tucker *et al.* 2001).

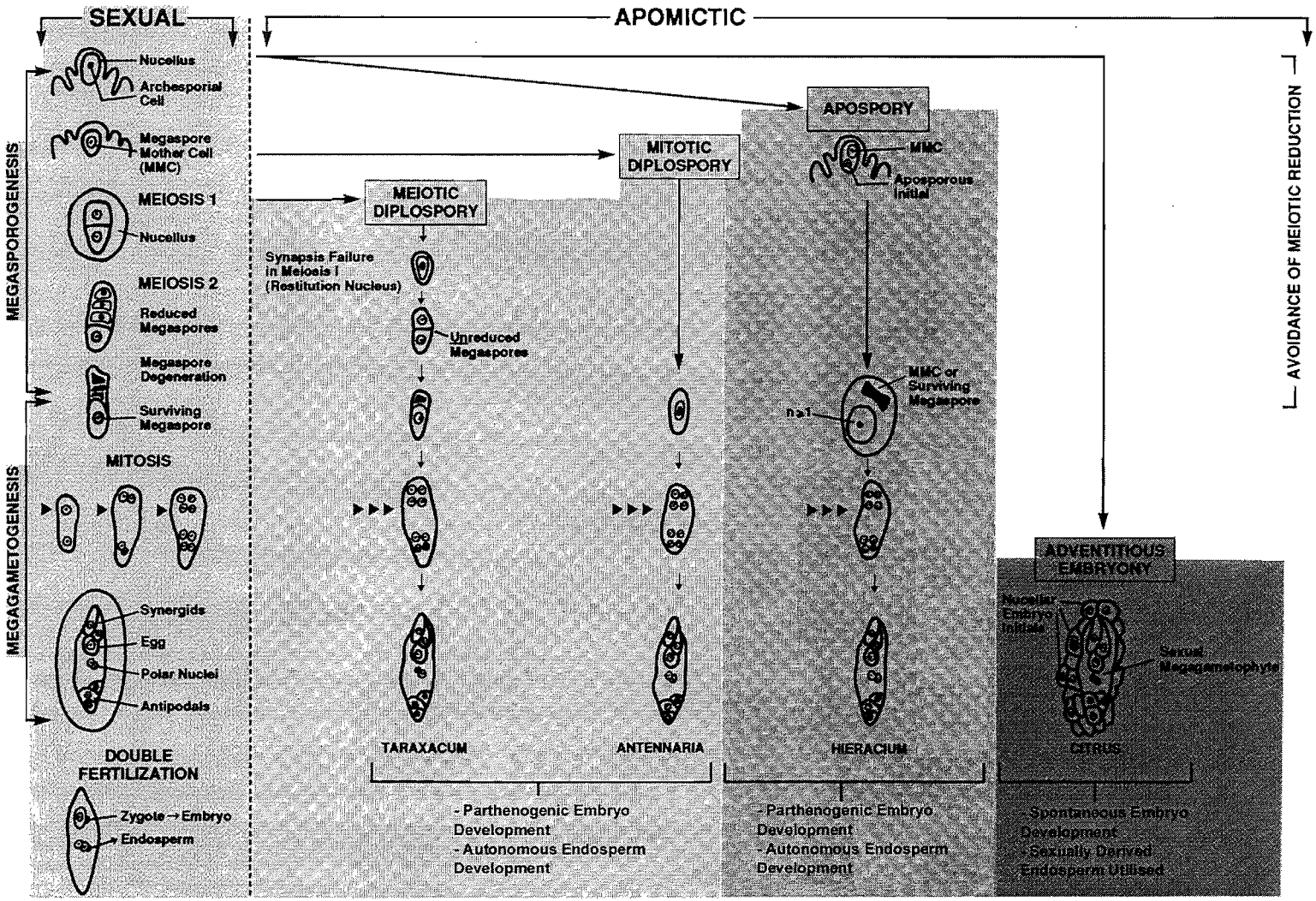


Figure 1.2.3 A Comparison of the three main Apomictic Pathways and Sexual Reproduction.
The example of *Hieracium* shown refers to *Hieracium* subgen. *Pilosella*. Apomixis in *Hieracium* subgen. *Hieracium* is of the diplosporous type (Modified from Koltunow 1993).

I.2.6 Mechanism for Sex in Apomictic *Hieracium* subgen. *Pilosella*

The earliest reports of apogamy in *Hieracium* spp. were formalised in Rosenberg (1906). This paper describes the presence of aposporous embryo sacs in *H. flagellare*. A later paper by the same author (Rosenberg 1907), identified the facultative nature of apomixis in the subgenus *Pilosella*, describing both hybrid and apomicticly derived progeny following a cross between *H. excellens* (maternal parent) and *H. aurantiacum* (paternal parent). Although this work described the embryology of this reproductive pathway, later works have produced more thorough treatments.

Skalińska (1971, 1973) examined the potential for facultative sexuality in *Hieracium aurantiacum,* and through embryological and cytological examination determined the mechanism for this. This is described in more detail in later work, particularly Koltunow *et al.* (1998). Examination of both *H. piloselliodes* and *H. aurantiacum* has determined that although aposporous embryo sacs are present, the "normal" meiotic division of the archaesporium and subsequent formation of the meiotic embryo sac is not interrupted. The aposporous embryo sac typically develops faster than the meiotic sac, and it has been suggested that this may be because of the time it takes to complete meiosis. As a consequence, the meiotic embryo sac is often displaced (see Figure 1.2.2). Sexual events in plants such as these rely on the sufficiently early development of a sexually derived embryo to displace the apomict one, and allow the formation of an outcrossed seed (Skalińska 1971, Koltunow *et al.* 1998). Embryological examination of many embryo sacs found that unreduced and reduced egg cells were accompanied by well developed endosperm, and also had the potential to be fertilised several days after the expansion of the florets (Skalińska 1973). The fact that *H. pilosella* can produce both amphi- and apomicticly produced seed in the same capitulum makes it an ideal model for investigating the role of the environment on the maintenance of apomixis.

I.2.7 Inter-fertility among *Hieracium* subgen. *Pilosella* species

A potential contributor to the high amount of variation in *Hieracium* subgen. *Pilosella* is the ability of the species to hybridise. It is clear from the literature that almost any of the species in this section can readily form hybrids with any other.

Hybrids between *Hieracium pilosella* and *H. aurantiacum* as mentioned earlier are very common in the wild where they are referred to as *H.* x*stoloniflorum* (Webb *et al.* 1988). *H. pilosella* is also known from Europe to hybridise with *H. peleterianum,* as well as *H. piloselloides* (Stace 1997, Koltunow *et al.* 1998). *H. caespitosum* is known to hybridise with *H. praealtum* and *H. lactucella* [*Pilosella* x*floribunda* (Wimm. & Grab.) Arv.-Touv.; in Stace 1997]. Glasshouse crossing experiments have also created successful hybrids between apomictic lines of *H. caespitosum* and *H. praealtum* (Chapman & Bicknell 2000).

Investigations into the chromosome number and reproductive systems of some species in *Hieracium* subgen. *Pilosella* have concluded that many of the species in this group are intermediate to each other in morphology. Although it is not certain that all of these taxa are of hybrid origin, the presence of some diagnostic characters, particularly cytological characters, indicate that this is the likely origin of much of the variation. It would also seem that backcrossing, or the participation of unreduced gametes may have played a role in the formation of this complex (Krahulcová & Krahulec 1999). The presence of many putative hybrid taxa in the Czech Republic (Krahulcová *et al.* 2001), where this has been extensively studied, shows the lack of reproductive isolation at the species level in this group.

I.2.8 The Importance of Seed in *Hieracium* subgen. *Pilosella* Populations

The ability to produce stolons facilitates the weed status of *Hieracium pilosella* in the high country of New Zealand. The significance of stoloniferous spread of *H. pilosella* was investigated by Makepeace (1985b) who concluded that only one percent of new rosettes were formed from seed, based on a comparison between the amount of single

rosettes found and those present in mats. This may underestimate of the importance of seed. Each mat of *H. pilosella* spatially separated from others by the maximum distance a stolon can elongate [average stolon length under field conditions has been reported as 22mm (Lamoureaux 1998)], has to initially be formed from seed. Seed is important as it represents the "long-range" dispersal function of *H. pilosella.* The fact that *Hieracium* spp. arrived in New Zealand as a contaminant of pasture seed from Europe, and has managed to colonise such a large area, is testament to its ability to spread by seed.

A recent paper by Rose and Frampton (1999) is the most thorough investigation of the magnitude of seed input into populations of *Hieracium* spp. in New Zealand. Seedlings were defined as single, non-flowering rosettes at least 15cm from any other plant of the same species. Densities of seedlings were recorded for tall and short tussock grassland. The number of seedlings for each type of site were 57.9 and 25.8 per 100 m2 respectively. As the definition of seedlings only applies to plants under one year of age, this represents a significant contribution to populations per annum.

This study is contrasted by Lamoureaux (1998), who in a survey of *Hieracium pilosella* at Mt John near Tekapo concluded that seed did not contribute to population recruitment over the season studied. Although this study was over two years, seedlings were only recorded in the first summer, and none survived to the second year of flowering. The number of seedlings found was extremely low, comprising only 35 individuals in the survey of 4256 plants during 1994 / 95. This would indicate the contribution of seed to populations varies between seasons, and site. The sites at Mt John used for this study represented a far more modified situation, and smaller geographic scale, than those examined by Rose and Frampton (1999).

Seed also represents the potential for sex in *Hieracium pilosella,* and the evolutionary escape from the "dead end" so often associated with apomicts (see Stebbins 1950). As suggested in the next section, seed represents the true reproductive potential of this species, regardless of whether the embryo is the product of sex or not.

I.2.9 Stoloniferous Propagation in *Hieracium* subgen. *Pilosella*, growth or reproduction?

The convention in many publications is to refer to the stoloniferous production of rosettes in species such as *Hieracium* subgenus *Pilosella* as a form of reproduction (see Makepeace 1981, 1985a, 1985b, Jenkins 1992). Historically, some have even referred to this as a form of apomixis (Winkler 1908) and a small subset of botanists maintain this today, although inexplicably they restrict the use of the term to vegetative reproduction in plants that are unable to reproduce sexually (Briggs & Walters 1997). This work restricts the use of the term to seed produced via parthenogenesis.

A new rosette is an example of modular growth, as is a branch on a tree, the difference being in stoloniferous taxa such as *Hieracium pilosella* the modules are less integrated and easier to define (Mogie 1992). The confusion begins in apomictic taxa when we have rosettes produced from seed that are genetically identical to those from stolons. In such cases, even defining what constitutes an individual is problematic.

Dawkins (1982) suggests a definition for reproduction, specifically that reproduction "permits a new beginning, a new developmental cycle and a new organism which may be an improvement, in terms of the fundamental organisation of complex structure, over its' predecessor". Although reproduction may not result in the improvement of the phenotype, the fact that there is potential for change is the important distinction. How an apomict would fit into Dawkins' framework is unclear; perhaps there would be a distinction between seed depending on the nature of its origin? Conversely, the majority of seed being no different from the parent may not be nearly as important as the fact that some may be significantly so. Mogie (1992) suggests a simpler model, which requires reproduction to be initiated by a single cell. This has some obvious flaws, particularly that the sporophyte and gametophyte would be declared different individuals, and in apomicts, identical ramets could be identified as either the same or different individuals whether they were produced from stolons or seed. Mogie (1992) concludes that we should not include vegetative propagation in reproduction, as it is not a direct competitor to sexual reproduction. It is this lack of competition that vegetative growth offers to

conventional sexual reproduction that relegates the production of rosettes by stolons to the realms of growth.

New Zealand publications have used rather arbitrary divisions between growth and reproduction in *Hieracium* subgen. *Pilosella*. Makepeace (1985b) follows a conventional definition of a "new" plant in *H. pilosella,* by considering a new rosette that had formed roots as being a new individual, and therefore a product of reproduction (see also Makepeace 1981, 1985a, Jenkins 1992). The reason Makepeace (1985b), and others, who adhere to this classification, gives for this definition is that at this stage of development it is unlikely for transport of water or photosynthates from the parent to the daughter rosette to be significant.

Stolon production in *H. pilosella* in New Zealand is significant, and one of the key factors of its invasiveness (Makepeace 1985b). The potential for evolution, however, is almost exclusively the domain of the seed. For this reason, and the fact that vegetative reproduction is not a competitor to sex (Mogie 1992), that this work will restrict the use of the term reproduction to seed events. This should not be confused with fitness, which is influenced by the acquisition of copies of a plant (and therefore the genome) of an organism. The division between growth and reproduction is much less defined in biology than between reproduction and fitness, and this work adopts the definition of reproduction in plants that at least includes the gametophyte at some level.

I.3 Potential Applications

The problematic nature of *Hieracium pilosella* in New Zealand has bought it to the attention of many different groups of people. Because of the unique reproductive system of this species, and the success it has had as an exotic invader, there are two particularly relevant practical applications to this research.

I.3.1 Apomictic Gene Technology

The transfer of the gene or genes for apomixis into important crop species could potentially revolutionise crop production. It has been suggested that the transferring of apomixis into crop species could be a more important advance than the adoption of semi-dwarf grain varieties (Vielle Calzada *et al.* 1996). At present, most of the important crop species worldwide use hybrid seed to increase yields. Hybrid vigour in some crop species can increase yields up to 81% (pearl millet, *Pennisetum glaucum*) (Hanna 1995), and often to be economically competitive, the use of such seed is necessary (Asker & Jerling1992). Maize (*Zea* spp.), wheat (*Triticum aestivum*), and sorghum (*Sorghum bicolor*) are all important crop species where hybrid origin is used to increase yearly yields. Some hybrid rice varieties have been shown to have a 20% increase in yield over conventional lines, but as hybrid seed is 5 to 10 times more expensive, it is not widely used (Koltunow *et al.* 1995). In most cases the hybrid is produced by inbreeding lines via the "double-cross" method, to maximise heterozygosity and therefore vigour. The transfer of apomixis to these species could potentially fix the heterosis effects of hybridisation. The elimination of pollination inefficiency or failure would also maximise yields and simplify production for many species (Bicknell & Bicknell 1999, Ramulu *et al.* 1999).

The drawback of many hybrid systems is that they either result in the sterility of the plant, making seed collection for future use impossible, or within a few generations the vigour has decreased to untenable levels via recombination. This makes purchasing hybrid crop seed necessary on a regular basis to maintain high yields. The transfer of

apomictic genes to the desired vigorous hybrid would overcome both problems, allowing the reuse of seed and ensuring high yields (Bicknell & Bicknell 1999).

Some species, such as hybrid wheat (*Triticum aestivum*), are particularly difficult to selectively breed as they require the use of cytoplasmic male sterility and restorer genes, whereas others, for example barley, (*Hordeum vulgare*) are self-fertilisers and therefore difficult to hybridise. The production of both these critical crop species would benefit greatly from the transfer of apomixis by fixing the effects of complex artificial breeding (Asker & Jerling 1992).

Although there have been numerous attempts to introduce apomixis into crop plants by selective breeding with close relatives, this has often resulted in plants with low seed fertility that are agriculturally unsuitable (Ramulu *et al.* 1999). The exception to this is probably pearl millet [*Pennisetum glaucum*] (Hanna 1995). This has fuelled interest in the identification of a gene or genes for apomixis, for insertion into a target species via biotechnological methods (Ramulu *et al.* 1999).

Increased efficiency through the use of apomictic cultivars

Crop production costs could be reduced if it became possible to use seed stock for species currently cultivated by tubers. The reduction in shipping, storage and planting costs due to the efficiency of using seed, as well as the avoidance of phytosanitary problems, will also result in advantages to the producers (Bicknell & Bicknell 1999, Ramulu *et al.* 1999). The adoption of apomictic grain crops could also increase mechanical harvesting efficiency by producing plants with more uniform heights, and greater synchrony in maturity (Hanna 1995).

Apomixis could also reduce the cost of hybrid crop production by preventing the need for progeny testing of hybrid cultivars for genetic stability of desirable traits, and potential strains would be immediately ready for appraisal as commercial plants. Outcross contamination is eliminated, and a greater range of germplasm may be utilised by crossing apomictic paternal parents against sexual maternal parents, making male sterility, and the need to maintain parental lines, obsolete (Hanna & Bashaw 1987, Ramulu *et al.* 1999). This would potentially decrease the amount of land required for the

production of hybrid seed, and by crossing apomicts to sexual progenitors, it would be possible to produce a range of genotypes with the same suite of desired traits. This may also allow faster responses from the breeders to market demands for particular characteristics, due to the ease of fixing desired variants. The ability to produce numerous cultivars with desired traits will potentially increase the diversity of crop stands to ensure yields, which may also avoid problems due to pathogens or epidemics (Ramulu *et al.* 1999). The use of transgenics would also be facilitated, as there would be no need for inbreeding to obtain transgenic homozygotes (Vielle Calzada *et al.* 1996). The potential to produce fixed hybrid crop lines from breeding with locally adapted cultivars could also be of benefit in less developed agricultural systems. At present, most hybrid varieties are developed for use in high input, resource intensive systems in developed countries. The ease of developing hybrid apomictic lines could transfer the advantages of an elite line to local, small-scale producers (Bicknell & Bicknell 1999).

Commercial considerations

Although apomixis allows farmers to retain seed for use in the following season, it is still possible for hybrid lines to be patented via marker inserts as a form of identification. It is thought that the advantages of purchasing commercial seed (guaranteed quality, treated and sized seed) would outweigh the relatively small cost of replacement, and most farmers would continue to buy from seed companies. The ability to retain seed of hybrid apomicts may be of benefit in third world countries, however, where it may be possible to increase food production by this method (Hanna & Bashaw 1987).

There have been concerns raised on the proprietary rights for apomictic crop production to be monopolised by some of the major life-sciences companies. The Bellagio Apomixis Declaration was tabled in 1998, in which many leading researchers in apomixis declared that apomixis technology must be available in developing countries (Bicknell & Bicknell 1999). Time will tell if such goodwill will be maintained in a commercial environment.

Currently, only a few commercial species possess apomictic reproduction, all of which are either natural apomicts or have closely related taxa that are apomicts to allow establishment of the trait via selective breeding. *Poa pratensis* (Kentucky bluegrass) and

Pennisetum ciliare [= *Cenchrus ciliaris*] (buffelgrass) have both been released as apomictic cultivars (Hanna 1995), and there are reports of facultative apomixis in *Sorghum bicolor* (sorghum) and *Pennisetum squamulatum* (pearl millet). *Citrus* spp. are often apomictic, and this has been utilised to produce genetically uniform, and virus free, rootstocks for commercial use (Hanna & Bashaw 1987).

Hieracium subgen. *Pilosella* as a model system for apomixis

There has been much interest in the isolation of apomictic genes, and some of this research has focused on *Hieracium* subgen. *Pilosella* spp. as a potential model (see Koltunow *et al.* 1998). The apomictic mode of *H. pilosella* is ideal for transferral to crop species as it combines the relative embryological simplicity of aposporous apomixis, with autonomous endosperm production, eliminating the need for pollination. The short generation time of the plant and the ease with which flowering can be induced under glasshouse conditions are also advantages for experimental investigation. The simple inheritance of this trait, and its' behaviour as a single dominant allele, are also favourable for use in crop species (Koltunow 1993, Koltunow *et al.* 1998, 2000). There is some evidence, however, for the presence of modifiers on the *Hieracium* apomixis gene, and this will also have to be addressed at the molecular level (Bicknell *et al.* 2000, Koltunow 2000, Koltunow *et al.* 2000).

Environmental plasticity and the application of apomixis technology

A neglected area of this research has been the environmental influence on the expression of apomixis in such species. The environment can play a role in the expression of apomixis in some species (Knox 1967), and may be important in that the prevalence of apomictic ovules in *Hieracium pilosella* is seemingly under physiological control (Skalińska 1971). If the expression of apomixis in *H. pilosella* is influenced by environmental conditions, this could have important implications for the use of such a system in cropping. This may identify limitations in the application of material from this species to apomixis programmes, or determine optimum conditions for the appraisal of the suitability of particular phenotypes.

Increasing the understanding of the role of the environment on the expression of apomixis in the model plant *Hieracium pilosella* will facilitate the development of this

plant as a potential gene donor, and allow us to better understand the effect of the environment on the breeding system of facultative apomicts.

I.3.2 Bio-control

Characteristics of bio-control programmes

The use of insect pests or pathogens to control weed species has been a major growth area in both evolutionary biology and agricultural study. The success of programmes attempting to control invasive plant species has been mixed, and different authors have drawn contrasting conclusions. Cullen and Hasan (1988) suggested that the variation in the plant species was of little concern in the success of such programmes. They assumed that if a plant was naturally highly variable then the pathogens of interest would have either a wider host range or a greater variety of strains than more conserved taxa. The shorter generation time of the pathogens was also thought to be a sufficient evolutionary advantage to prevent the establishment and perpetuation of resistant host types. It is interesting, however, that the example they most often cited was of *Chondrilla juncea* (skeleton weed), and the highly specific control agent, *Puccinia chondrillina.* Although three "forms" of *C. juncea* are found in Australia as invasive weeds, only one was susceptible to strain IT32 of *P. chondrillina* that was introduced as a control agent. Control of this species seemed to be highly influenced by the host–species interaction, despite the conclusions drawn from this work. In contrast, Davelos *et al.* (1995) found little effect of host genotype in *Spartina pectinata* on infection with *Puccinia seymouriana* and *P. sparganioides* over moderate (4.5 km) spatial scales.

Barrett (1992) however, views host population structure as a key to successful control of a weed. Using *Eichhornia crassipes* (water hyacinth) as a model, Barrett (1992) suggests that the clonal vegetative propagation of this species results in a relatively uniform population structure, and therefore it is imperative to find a pathogen with good efficacy on particular genotypes if control is to be effective. Knowledge of population structure in these cases in essential for the success of a programme. It was also noted that up to the present, by far the most focus is centred on the range of genetic variation in the pathogen, with almost no studies into the levels of variation in the host. For effective control it is

probably most beneficial to have information on both host and pathogen population structures (Barrett 1992). It is also important to be aware of the potential for the generation of future population variation in each group of organisms. It has also been pointed out that more successful bio-control programmes have been centred on species that reproduce asexually than sexually (Burdon & Marshall 1981, Cullen & Hasan 1988).

In all cases of pathogen or insect control agents for weed species the aim of such programmes is not the eradication of the target, but its reduction to a level that reduces its environmental or economic impacts to a level that is acceptable for the particular land use. In a lot of cases eradication may be desirable, but by their nature, bio-control agents will not force a host into extinction (Barrett 1992).

The current *Hieracium* bio-control programme

Due to the impact *Hieracium pilosella* has had in the high country of New Zealand there is an interest in the potential control of this organism. The main control option examined so far has been natural pests or pathogens of *H. pilosella*, usually introduced from Europe. Initially, because of its' supposedly clonal nature, a host-specific rust fungus, *Puccinia hieracii* var. *piloselloidarum*, was considered a good candidate for control (Jenkins 1995). This has proven ineffective, and we would now expect that this is due to the high levels of intra-population variation in the host. Subsequently, other strains of the fungus have been introduced in an effort to increase the range of this pathogen (Syrett *et al.* 2001). The conclusion drawn from this was that several different genotypes of *H. pilosella* are present in New Zealand, and due to the specificity of the rust, only a few will be affected (Morin & Syrett 1996). Further studies of genotypic variation in New Zealand populations of *H. pilosella* have discovered high levels of variation (Chapman *et al.* 2000 as *Pilosella officinarum*), and it appears that this variation limits the effectiveness of the rust fungus under glasshouse conditions (D. Trumm & H. Chapman *unpubl. data*). The presence of resistant genotypes at the point of release minimises the potential population decrease in the host by a control agent (May & Anderson 1983, Anderson & May 1986). This has probably been the case for control of *H. pilosella* with *P. hieracii* var. *piloselloidarum* in New Zealand.

The Uredinales, which includes the genus *Puccinia*, are common plant parasites, and it has been postulated that there has been considerable co-evolution between the fungi and their hosts (Rice & Westoby 1982). *P. hieracii* var. *hypochoeridis* and var. *hieracii* have both been present in New Zealand for some time, and have been identified attacking several different hosts (Morin & Syrett 1996). This suggests that *P. hieracii* var. *piloselloidarum* may be more closely evolved to its' host species than usual, even in this highly specific group.

Puccinia spp. have in some cases proved to be some of the most effective bio-control agents to date. Their excellent host specificity, even to the genotype level, means that in populations with largely clonal population structures they can be extremely effective. The classic example of this was the control of *Chondrilla juncea* with *P. chondrillina* in Australia, as mentioned earlier. The population density of the "A" form of this plant when attacked by the IT32 strain of *P. chondrillina* fell to one hundredth of its previous level, approximately the density in its' native Europe (Cullen & Hasan 1988). The low level of genotypic variation present in these populations, even for an apomict, is thought to be the explanation for the outstanding success of this particular programme (Barrett 1992).

The identification of the environmental conditions that promote sexual reproduction in *Hieracium pilosella* in New Zealand could have applications when planning potential bio-control programmes. Increased testing of agents on a range of host genotypes, and recognising the potential for change in host genotypes over relatively short time frames under certain conditions, may indicate what sort of organisms will be the most successful. Recognising the dynamic nature of *H. pilosella* population structure may improve future bio-control programmes. To date, releases of the rust agent have been relatively random, with landowners having the responsibility to spread the pathogen (T. Jenkins *pers comm.*). This can make assessing the impact of an agent under different conditions impossible, and provides little information on the effect of population structure or potential micro-evolution. Better understanding of the reproductive mode and population structure of this species will indicate whether a potential control agent has broad enough specificity to be a worthwhile introduction.

Other bio-control agents for *Hieracium pilosella* destined to be released in New Zealand may increase the effectiveness of the programme due to a cumulative effect. A parthenogenetic gall-wasp, (*Aulacidea subterminalis*), a gall midge (*Macrollabis pilosellae*), a moth (*Oxyptilus pilosellae*) and a syrphid larvae (*Cheilosia praecox*) have all been identified as potential control agents. The gall wasp and gall midge are both restricted to *H. pilosella,* whereas the other two agents are specific to *Hieracium* spp. (Syrett *et al.* 1996). These will be less specific than the rust fungus, and the differing host-plant genotypes will hopefully not have such an impact on the effectiveness of these agents. Interpretation of the effects of these control agents and future development of control programmes may be further facilitated by a better understanding of population dynamics. The identification of factors that can be controlled by management, such as fertiliser levels, and their effects on reproduction, may indicate an optimal strategy of bio-control supplemented by the most beneficial agricultural practices.

Alternatives to bio-control

Other methods of control for *Hieracium pilosella* have also been considered. Over-sowing and fertilisation can provide a recovery of pastureland to levels that allow viable grazing. Legume species such as alsike (*Trifolium hybridum*) and white clover (*T. repens*) are particularly suitable for this method (Scott *et al.* 1990). This does require, however, high levels of fertiliser application and irrigation, which is often not financially viable over large areas. This method is not suitable for native grassland areas as such modification destroys their conservation value.

There have been herbicide trials for the control of *Hieracium* spp. but environmentally acceptable variants have had a poor mortality rate. The cost of herbicide application is also around $110-200/ hectare, making them only really suitable for spot control or small-scale spraying. The manipulation of grazing regimes has also been attempted, but the results of these trials have been inconclusive or disappointing (Scott *et al.* 1990).

Alternatives to bio-control for *Hieracium* spp. have proven to be largely unworkable, usually due to financial cost. The lack of specificity and lethality of other methods has left bio-control as the method of choice for the control of *Hieracium* spp. in this country.

Due to the high cost of determining the suitability of a control organism, it is imperative that the maximum possible decrease in *Hieracium* spp. is effected with each release.

I.4 Evolutionary Selection for Asexual Reproduction, a Theoretical Framework.

I.4.1 The Maintenance of Sex

Sexual reproduction, although prevalent in higher organisms, is a comparatively expensive method of reproduction. Population demographic models predict that sex has a two-fold cost when compared to asexual reproduction. Why organisms would persist in reproducing sexually when other, seemingly more efficient, methods are available, is an ongoing paradox in evolutionary biology.

The reason sexual reproduction is so costly is due to the "cost of meiosis", or "cost of males" (Williams 1975, Maynard Smith 1978). In asexual species the progeny possess the entire genome of the parent and the number of offspring is directly proportional to the fitness of the individual. Sexually reproducing (amphimictic) individuals also pass their genome to the next generation, but it is only a proportion of this genome that is inherited by any one offspring. In most amphimictic organisms, the gametes are reduced to n from 2n, and therefore typically contain only one half of each parent's total genome (Ridley 1993). This confers a two-fold fitness advantage to those that avoid sexual reproduction. Alternatively, this can be viewed as sexual individuals wasting resources investing in males when they could potentially be producing parthenogenetic females. This cost, whether it be the modelled as the "cost of males" or "cost of meiosis" can be represented diagrammatically (See Tables 1.4.1, 1.4.2).

It is clear that it is far more efficient for organisms to reproduce asexually to preserve the integrity of their genome, and to maximise their fitness (Lloyd 1980), and in the majority of eukaryotic taxa this is the case (Dacks & Rodgers 1999). Why then is sexual reproduction so prevalent in such a range of different organisms, and why would a potential obligate apomict retain some level of sexual reproduction?

Table 1.4.1 Sexual Vs Apomictic Reproduction: The Cost of Males.
Scenario: One apomictic individual and two sexual individuals (one male, one female) form a founder event. If each female gives rise to 4 progeny, within a few generations, parthenogenetic individuals become far more numerous, due to the cost of males. This model predicts that by generation 10, 99.6% of all individuals will be apomicts.

Generation	Sexual	Apomictic	% of Population Asexual*
1	M F	F	33.33%
2	M F M F	F F F F	50%
3	M F M F M F M F	F F F F F F F F F F F F F F F	66.67%
4	M F M F M F M F M F M F M F M F	F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F F	80%

*Assuming previous generation dies at the beginning of each generation.

Table 1.4.2 Sexual Vs Apomictic Reproduction: The Cost of Meiosis
Scenario: Ignoring the cost of producing males, it is still clear that it is in the best interest of an individual to reproduce asexually. This shows the relative transfer of the parental genome to the next generation. F = Relative Fitness. X is used, as the sex of the progeny is irrelevant to this model, and each individual contributes to 2 offspring.

Generation	Sexual	Number of copies of parental genome in F_1 generation.(F)	Apomictic	Number of copies of parental genome in F_1 generation.(F)
1	X	NA	X	NA
2	X X	0.5+0.5 = 1	X X	1+1 = 2

Weismann in the late 1800s was one of the first to identify the paradox. He stated in 1889 that "The significance of amphimixis cannot be that of making multiplication possible, for multiplication may be effected without amphimixis in the most diverse ways - by division of the organism into two or more, by budding, and even by the production of unicellular germs." (In: Ridley 1993). Although the original explanation provided by Weissman for this has been superseded by later explanations (although see Burt 2000), this has still remained problematic. The presence of sexual reproduction becomes even more perplexing in taxa such as *Hieracium pilosella,* which are predominantly asexual, but retain amphimixis at some level.

Chapter II. QUANTIFICATION OF SEXUAL REPRODUCTION IN NEW ZEALAND POPULATIONS OF *HIERACIUM PILOSELLA*

II.1.1.FIELD HYBRIDISATION: CROSSING NATURAL POPULATIONS OF *HIERACIUM PILOSELLA* WITH *H. AURANTIACUM.*

The facultative nature of apomixis in *Hieracium pilosella* has been discussed in several works (Rosenberg 1906, Skalińska 1971, 1973, Koltunow *et al.* 1998), but the frequency of sexual reproduction in predominantly apomictic *H. pilosella* under field conditions is unknown. Sexual reproduction in facultative apomictic populations is one of the most commonly suggested mechanisms for the generation of the complex population structures often observed in these species (for a review see Chapter III.1). This chapter aims to quantify the potential for sexual reproduction in New Zealand populations of *H. pilosella* under field conditions.

Quantifying the frequency of sexual reproduction in apomictic taxa

The quantification of the frequency of sexual reproduction in facultative apomictic populations has often proven problematic. Numerous experimental methods have been employed, some of which are more appropriate for certain investigations than others. These methods can be divided into several categories:

Cytological investigation of putative hybrid progeny

Gadella (1987) attempted to measure the frequency of sexual reproduction in pentaploid *Hieracium pilosella* by examining the ploidy level of offspring following pollination with a tetraploid. As all the offspring examined were pentaploid, he concluded that all progeny were the result of apomixis. This method involves assumptions being made on the reduction levels of gametes produced by both parents, and is only suitable in cases where meiosis is predictable. In *Hieracium* subgen. *Pilosella* meiosis is often irregular (Bicknell *et al.* 2000) which may make the application of this method unsuitable. More recent works have employed flow cytometry to determine the ploidy of putative hybrid

individuals (Tas & van Dijk 1999), and this may have potential for further application (see Chapter II.3).

Cytological investigation of the ovule

Several studies have relied on cytological examination of embryo sacs to determine the potential for sexual reproduction (Knox & Heslop-Harrison 1963, Knox 1967, Evans & Knox 1969, McWilliam *et al.* 1970, Burton 1982), however this is very labour intensive, and not always an accurate measure. Although the presence of non-apomictic embryo sacs can be visualised, it is an assumption that these are actually viable and able to produce outcrossed progeny. Studies in *Taraxacum* have found low frequencies of reductional meiosis in apomicts, but most likely at a much higher rate than was observed to reproduce sexually (van Baarlen *et al.* 2000). This method has also been applied to *Hieracium* subgen. *Pilosella* but the sample sizes of these studies have been limited (Koltunow *et al.* 1998, Bicknell *et al.* 2000). This method can potentially produce highly accurate results in some groups, and provide a lot of information on the nature of embryo sac formation, but is very labour intensive.

Molecular approaches

Recent work has employed microsatellite markers to quantify the frequency of sexual reproduction in *Hyparrhenia diplandra* (Durand *et al.* 2000), and while this provides accuracy, it also requires high labour input. Isozyme electrophoreses has also been employed to quantify the frequency of outcrossing in facultative apomictic *Antennaria media* (Bayer *et al.* 1990). Neither of these methods is particularly suited to population level studies where large sample sizes are mandatory.

A more novel technique was employed by Tas and van Dijk (1999), and van Dijk *et al.* (1999) to detect hybridisation events in *Taraxacum.* They selected maternal parents that were homozygous for a unique isozyme locus, and used only this single enzyme system to detect hybridisation events. Although in this case the maternal parents were obligate sexual and the paternal parent apomictic, if it was possible to find such a marker in an apomictic species this could potentially be a useful method. Finding such a marker in an apomict is more problematic than for an obligate sexual however, as it is relatively easy to select true breeding individuals from backcrosses.

Several alternative molecular methods are available for the detection of sexually produced progeny in facultative apomicts. Antonius and Nybom (1995) used a combination of methods to distinguish sexually produced progeny in facultative apomictic *Rubus* spp. from those produced via apomixis or automixis. Morphologically distinct categories of plants were fingerprinted using the M13 probe, and the profiles compared to the putative parents to determine if morphologically intermediate progeny were in fact the result of recombination. This was necessary due to the presence of automixis in this group, allowing for the generation of new genotypes asexually, necessitating a marker other than morphology to identify hybrid origin. Although this study did not aim to identify the rate of recombination in a facultative apomict, this method did prove to be suitable for this task. A similar study, van der Hulst *et al.* (2000), used AFLPs to demonstrate that recombination occurs in triploid apomictic *Taraxacum officinale.* This was estimated by the occurrence of unique individuals in the predominantly clonal populations. While this method does not quantify the actual rate of recombination in the organism, there is perhaps potential to extrapolate these data to rates of recombination. These approaches still have the problem of very high labour input if large sample sizes are desired. Espinoza *et al.* (2002) used RAPDs to test progeny of facultative apomictic *Paspalum notatum* following pollination at different stages of capitula development. Although the sample sizes were sufficient for the investigation of pollination efficiency, this is far more labour intensive than other, non-molecular, methods and again, not suitable for the numbers required for population based studies.

Loss of reproductive output in the absence of pollen

Some researches have attempted to quantify the frequency of sexual reproduction in apomicts by comparing the number of seed produced following pollination with those in the absence of pollen. A study has attempted to use this method to quantify the frequency of sexual reproduction in facultative apomicts of *Hieracium* and *Taraxacum* (Kashin & Chernyshova 1997), but this is generally not an accepted method. The amount of unfilled seed is largely dependent on environmental conditions, and in the glasshouse within even a single genotype variation can be large (see Chapter V).

Ovule characteristics and secondary compounds

Examination of callose (β1-3 glucan) deposition during megaspore formation has also been used as a measure of sexual reproduction in some apomictic taxa. The presence of β1-3 glucan in the walls of megaspore mother cells is used to indicate sexual events in predominantly apomictic individuals, especially in diplosporous taxa where this is absent during apomictic embryo formation (Tucker *et al.* 2001). β1-3 glucan can be detected after clearing the ovule material and staining with decolourised analine blue (Peel *et al.* 1997). This method is not suitable for *H. pilosella* as alterations in callose deposition are not related to differences in embryology between sexual and apomictic embryo development. Although there are differences in callose formation in apomictic and sexual plants, the presence of a megaspore tetrad in an ovule with Polygonum-type callose deposition does not mean the embryo will be produced via sex. This structure is often observed before the appearance of aposporous initials, which in most cases displace the expanded megaspore (Tucker *et al.* 2001). Although it is possible to see the displacement of the megaspore during aposporous initial development, this is not a practical method for determining the level of facultative sexuality in this species.

Hybrid characteristics as a marker for sex

In this study, to determine the frequencies of sexual reproduction in field populations of *Hieracium pilosella,* individual capitula will be artificially pollinated with *H. aurantiacum*, and the morphological characteristics of this species used as a natural marker. As mentioned in chapter 1.2.7, *H. pilosella* and *H. aurantiacum* freely hybridise, the natural hybrid between the two being known as *H.* x*stoloniflorum.* The hybrid is easily recognised by gross morphology. The key morphological differences between the species are floret and leaf colour, density of hairs on the abaxial surface of the leaves, and number of inflorescences per flowering stalk. Involucral bract hair arrangement is also a useful character (Webb *et al.* 1988).

The use of a hybrid marker allows a direct measure of the amount of sexual reproduction without the use of molecular or cytological methods. *Hieracium aurantiacum* has been identified as a particularly good pollen donor when crossed to other members of subgen. *Pilosella,* minimising mentor effects and providing clear heritable, morphological traits to identify hybrid progeny (Krahulcová *et al.* 1999).

Although it is generally thought that *H. pilosella* is self-incompatible (e.g. Gadella 1984, 1987, 1991a), there is some evidence for selfing occurring in this species via mentor effects (Krahulcová *et al.* 1999, R. Bicknell *pers com*). The presence of foreign pollen on the stigma can breakdown the biochemical self-incompatibility, allowing self fertilisation. The frequency of this is unknown, but Krahulcová *et al.* (1999) found that it was minimised when using *H. aurantiacum* as a pollen donor.

The use of a pollen donor with marker characteristics makes it possible to assay the potential for sexual reproduction of a population efficiently in regard to both time and cost. Genetic and cytological methods are also employed in this study, however, as they are powerful techniques to confirm the origin of the resulting hybrid progeny (see Chapters II.2 and II.3 respectively).

This approach has been used in the past, but always under glasshouse conditions (see Krahulcová *et al.* 1999, van Dijk *et al.* 1999, Tas & van Dijk 1999, Chapman & Bicknell 2000, Krahulcová & Krahulec 2000). Glasshouse based studies by their nature do not often accurately reflect the natural growing conditions of the plants. *H. pilosella* is most commonly found in the field where soils are depleted, rainfall is low and high grazing pressure exists (Hunter 1991, Connor 1992). To understand the potential for the sexual reproduction of this plant in the field we need to determine the reproductive output under such conditions. The use of several field sites with different conditions allows the influence of the environment on apomixis to be quantified. This will be discussed in chapter IV.1.

II.1.2 MATERIALS AND METHODS

Field sites and sample sizes

Seven sites were chosen to represent a range of environmental conditions and habitat, primarily altitude, rainfall, and vegetation. A general site description can be seen in table 2.1.1. Further site descriptions and a map of site locations are presented in Appendices 1.1 and 1.2 respectively.

Table 2.1.1 Field site Parameters, 1998–2001

	Altitude (M)	Soil type	Landscape	Co-ordinates
Cave stream	700	Yellow-brown earth	Limestone river terrace	S 43° 10.960' E 171° 44.350'
Dracophyllum flat lower	790	Yellow-brown earth	Intermontane valley	S 43° 09.005' E 171° 44.048'
Dracophyllum flat upper	810	Yellow-brown earth	Intermontane valley	S 43° 09.005' E 171° 44.047'
Cass flats	580	Yellow-brown earth	Gravel outwash	S 43° 02.173' E 171° 45.691'
Chilton valley	740	Yellow-brown earth	Eroded valley	S 42° 02.223' E 171° 46.336'
Little river	650	Bossu	Hilltop	S 43° 66.100' E 172° 46.500'
Redcliffes station	600	Yellow-grey earth	Gravel outwash	S 43° 23.156' E 171° 31.027'

Not all sites were assayed each year, and samples sizes at the sites varied, due to seasonal climatic variation. Site use and samples sizes by year can be seen in Table 2.1.2. The Cave stream site was not assayed during the 1999 / 00 season due to the abortion of all capitula at this site following extreme drought conditions. The Redcliffes station site was substituted as this site had two potential pathogens present, *Erysiphe* spp. (powdery mildew) and *Puccinia hieracii* var. *piloselloidarum* (rust fungus). A site with potential pathogens present was chosen to attmept to determine if these played a role in the reproductive mode of *Hieracium pilosella.* The Redcliffes station site was not assayed during the 2000 / 01 season as it was possible to again utilise Cave stream.

Table 2.1.2. Number of capitula pollinated at the field sites, 1999-2001

Year	Cave stream	*Draco* flat lower	*Draco* flat upper	Cass flats	Chilton valley	Little river	Redcliffes station	**Total**
1999	11	8	8	18	24	50	0	**119**
2000	0	13	16	25	28	30	8	**120**
2001	17	18	25	19	25	21	0	**125**
Total	**28**	**39**	**49**	**62**	**77**	**101**	**8**	**364**

Dates for the covering of inflorescences, pollination, and collection of seeds can be seen in Appendix 2.

Field pollination and glasshouse propagation

The artificial pollination method consisted of covering each capitulum bud with a bag of nylon insect cloth (580μm mesh) measuring 110mm X 80mm to exclude all pollination vectors. To support the bag, a wire loop with a diameter of 40mm on an upright cut to the correct length for the scape was placed over the inflorescence. The bottom opening of the bag was closed with adhesive transfer tape (3M™ Scotch 969) with a gap to allow the scape to elongate (see Figure 2.1.1). Once all the florets of the inflorescence had opened (capitula anthesis) the bag was removed and the capitulum gently rubbed with two inflorescences of *Hieracium aurantiacum* to effect cross-pollination. This resulted in an overabundance of pollen, and assured no pollen limitation occurred. Plants used as pollen donors were from a population of *H. aurantiacum* from Porters Pass (2n = 3x = 27) [referred to from this point as P2] and A 3.4, an aneuploid accession from central Otago (2n = 3x + 4 = 31) which is known to produce high levels of viable pollen (Chapman & Bicknell 2000). P2 was only used as a pollen donor during the 1998 / 99 season, the following years A3.4 was used exclusively as this accession was easier to maintain under glasshouse conditions. Once pollination was completed, the inflorescence was covered with the same bag arrangement as before. This was left in place until the seed had set, at which time the inflorescence was harvested. Details of this methodology are described in Houliston and Chapman (2001).

A trial of the effect of the pollination enclosures on capitula development was carried out prior to the 1998 / 99 field season. Details of this can be seen in Appendix 3.1 (Table A3.1.1).

Germination of the seed was achieved following the method of Bicknell (1994b). This method was chosen as it is known to give the highest possible germniation rate, and is superior to alternative methods for *Hieracium pilosella* (cf. Makepeace 1985a). Seeds collected following artificial pollination in the field were surface sterilised for 50 minutes in a solution of 1 % sodium hypochlorite and 0.1% Tween 80 detergent. They were then sown on an agar media [2.5% MS Macro 20x, 0.5% MS Micro 200x, 0.5% MS Iron

200x, 0.5% MS Organics 200x, 30g/l 3% Sucrose, 7.5g/l of Agar, final pH of 5.8 (Murashige & Skoog 1962)]. The seed was then placed in a growth chamber at 22° C with a 16 hour photoperiod. Makepeace (1985b) states this is the optimum for both the total number of seeds germinating, and the time to 50% germination. All samples were left for at least twenty-eight days before transfer to a mist propagation area. The percentage of viable seed was recorded and the seedlings transferred to potting mix with 3-4 mm perlite on top. They were later transferred to 50 mm square pots with potting mix containing slow release fertiliser. The plants were grown to flowering stage and the flower morphology recorded. Characterisation of the hybrids was carried out using several different methods (see also Chapters II.2, II.3). Plants were examined for morphological characters known to occur in *Hieracium pilosella* x *H. aurantiacum* hybrids.

Sample numbering

All individual crosses from the three years of the field study were assigned a four-digit number. The first number refers to the season: 1- 1998 / 99, 2- 1999 / 00, 3- 2000 / 01, the remaining three digits to the specific sample. A "p" suffix indicates the maternal type produced via apomixis. Aberrant (putative hybrid) individuals had sequential letter suffixes (i.e. "a" "b" "c"...). For example, 2051p is the maternal type of sample 51 in field season 2; 1117a is an aberrant (putative hybrid) individual from sample 117 in field season 1.

Figure 2.1.1 Pollination enclosures on *Hieracium pilosella* at the Cass flats field site, 1999.

Pollination enclosures were placed on the plants prior to capitulum opening, at the bud stage that can be seen in the foreground. Enclosures were usually placed at greater intervals than is illustrated here.

Morphological Analysis

Morphological characteristics for a subset of both putative hybrid and apomictically derived progeny from the 1998 / 99 field season were recorded for several key diagnostic characters. The individuals measured can be seen in Figure 2.1.8, and were chosen to represent the six field sites and range of morphology observed (n = 39). Detailed morphological analysis of the putative hybrid progeny was only carried out for the 1998 / 99 season (see Houliston & Chapman 2001).

The morphological characters used to discriminate between aberrant (putative hybrid) and non-aberrant (apomictic) individuals can be seen in Table 2.1.3. Multistate characters were scored as denoted in brackets.

Table 2.1.3 Morphological characters used in the determination of hybrid origin of putative individuals, 1998 / 99

Capitula characteristics			
Colour	pale yellow (1)	light orange (2)	bright orange – dark orange (3)
Rosette Leaves			
Dimensions			
Mean length (mm)	Average of 10 measurements per plant		
Mean width (mm)	Average of 10 measurements per plant		
Mean length/ width ratio	Calculated as average length / average width		
Abaxial surface			
Stellate hair density	dense (1)	many (1)	few (3)
Involucral Bracts			
Mean glandular hair lengths (μm)	Average of 20 measurements per plants		
Achenes			
Dimensions (mm)			
Length	Average of 10 measurements per plant		
Width	Average of 10 measurements per plant		

Dimensions of the achenes and glandular hair lengths were measured using the image analysis programme, Metamorph (Version 4.0, Universal Imaging Corporation). A MTI Dage CCD 72EX digital camera (756 x 581 pixel resolution) with a 55 mm Nikon macro lens was used to collect images, and the scale calibrated using Metamorph. The paternal parent *H. aurantiacum* and *H.* x*stoloniflorum*, a natural hybrid between *H. pilosella* and *H. aurantiacum,* were also included in the analysis for comparison to the putative hybrids.

Analysis of morphological data was performed using Multi Variate Statistical Package (MVSP Version 3.1a) (Kovach Computing Services 1999). Principle Co-ordinate Analysis (PCO) with Gower's similarity measure was chosen as it can use a variety of different character types to construct a similarity matrix. PCO specifically was chosen as distances or similarities are measured between the cases directly, rather than the variables as in Principle Component Analysis (PCA), and the eigenvectors represent the scores for the cases. Because of this, it is much more robust when there are more variables than cases, and the PCA method becomes unsuitable. The disadvantage is that no measures of explained variance are given for variables, and it is not possible to assign importance to characters. Following the construction of a similarity matrix, a two-dimensional plot of vectors was used to position both parents and the hybrid progeny in morphological space.

The calculation of the Gower (1971) coefficient:

$$S_{ijk} = 1-(|X_{ik} - X_{jk}| / R_k)$$

Where: X_{ik} is the score of individual i for character k,
X_{ij} is the score of individual j for character k,
R_k is the range of character k.

The number of putative hybrid progeny were recorded for each sample, and collated for each of the six sites per season. Detailed analysis of site, year, and environmental variation in frequencies is discussed in chapter IV.1.

Ploidy level of progeny was also examined, and the known chromosome number of the male parent compared to that of the maternal parent and the apomictic offspring. See chapter II.3 for details of techniques and the results of ploidy level determination.

Measuring the inheritance of sexual reproduction

The reproductive mode of the putative hybrid progeny from the 1999 / 00 and 2000 / 01 field seasons was determined following the decapitation of the capitulum bud. Hybrids that formed filled seed were deemed apomictic, those producing only empty seed, sexual, following the method of Richards (1991) (see Figure 1.2.1).

II.1. 3 RESULTS

Abortion frequencies and seed production

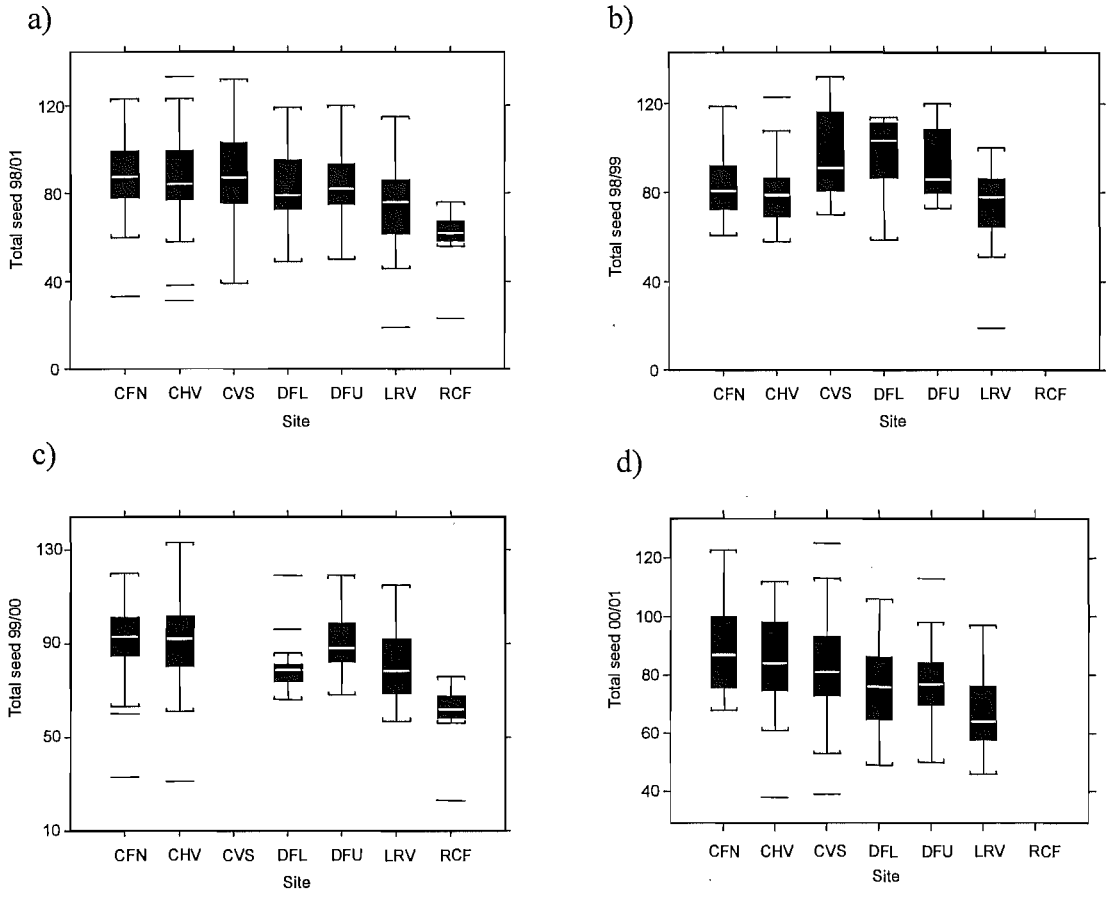
Abortion frequencies varied considerably, with a high degree of capitula failure at the *Dracophyllum* flat lower and Cave stream sites. Levels of capitulum success at the field sites varied considerably over the three field seasons (Appendix 3.2, Tables A3.2.1a-c). Abortion frequencies of capitula were highest in the 1999 / 2000 season, with 28 of the 120 samples failing to develop to maturation. This is most likely due to the varying climatic conditions and eventual drought at all sites during this summer. The 1998 / 1999 summer had by far the least abortion and greatest seed production, with the highest proportion germinable (see Figure 2.1.4).

Seed production at the field sites varied over the three seasons, with most sites having significantly different levels of seed production to others. Seed count data were square root transformed before analysis to remove the underlying Poisson distribution (Howell 1992, Zar 1996). Where there were significant differences between the seed production at sites (see Figure 2.1.2-4), there was no predictable (geographic, altitudinal, vegetation type) pattern, indicating that there is a high degree of variation in this trait over environmental and geographic gradients. Variation between sites was greater in general than between years, particularly in 1998 / 99. This pattern is also reflected in the frequency of sexual reproduction (see Table 2.1.5a-c). Total seed production was also

significantly different between years, and sites (Figure 2.1.2). There were also significant differences in filled (Figure 2.1.3) and viable (Figure 2.1.4) seed production with both year and site effects. That total seed production was significant between sites, and also years, indicates that genotype of the plants or site characteristics as well as between-year climatic variation determines the total seed production at a site. This was also reflected in the filled and viable seed production, with both site and seasonal effects being highly significant (see Table 2.1.4). There was considerable variation among the number of crosses performed at each site in the three years surveyed (Table 2.1.2). These differences were largely due to the phenology of the plants, and the environmental conditions at the sites. High levels of abortion also skewed the sample sizes, particularly at sites where obligate sexual plants were present.

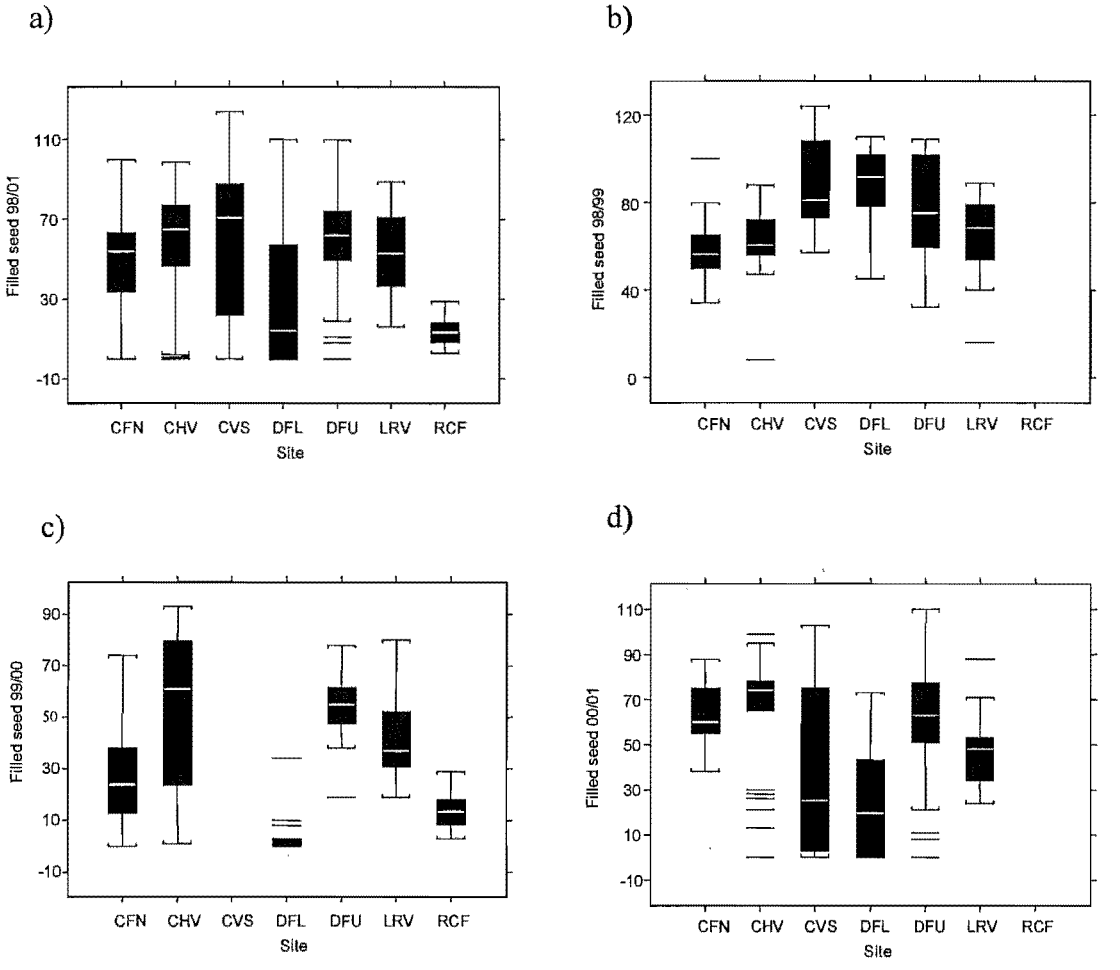
Obligate sexual plants often had lower seed output than facultative apomicts, although there was considerable variation in this trait over the three summers (see Appendix 2).

Figure 2.1.2 Total seed production by site, all seasons combined, and by season. [Site key: CFN – Cass flats, CHV – Chilton valley, CVS – Cave stream, DFL – Dracophyllum flat lower, DFU– Dracophyllum flat upper, LRV – Little river, RCF – Redcliffes station].



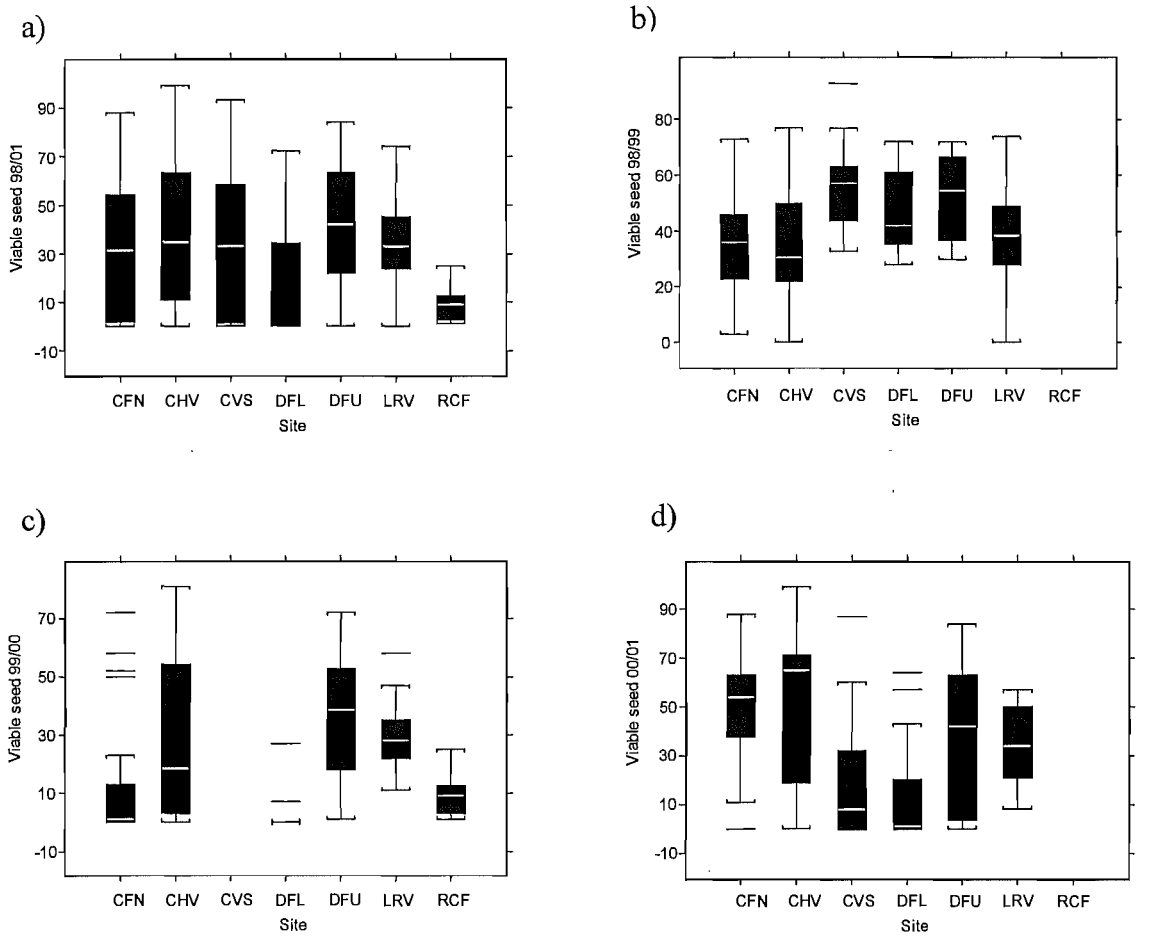
Data are presented in the form of box plots, sometimes referred to as box and whisker plots. The white line in the middle of the "box" represents the median of the data, whereas the limits of the box represent the upper and lower quartiles of the data distribution, and therefore the vertical size of the box represents the inter-quartile range. The whiskers, appearing as "error bars" extending from the box, extend to either the upper or lower "adjacent value". The lower adjacent value is defined as the smallest value greater than or equal to the lower fence, which is defined as the lower quartile minus 1.5 times the lower quartile value. The upper adjacent value is defined as the largest value less than or equal to the upper fence, defined as 1.5 times the upper quartile value. Single lines lying outside the whiskers represent outlying values, i.e. those that lie outside the fences (Howell 1992).

Figure 2.1.3 Filled seed production by site, all seasons combined, and by season. [Site key: CFN – Cass flats, CHV – Chilton valley, CVS – Cave stream, DFL – *Dracophyllum* flat lower, DFU– *Dracophyllum* flat upper, LRV – Little river, RCF – Redcliffes station]. a) Filled seed all seasons, b) Filled seed 1998 – 1999, c) Filled seed 1999 – 2000, d) Filled seed 2000 – 2001.



A description of the interpretation of box plots can be seen in Figure 2.1.2.

Figure 2.1.4 Viable seed production by site, all seasons combined, and by season. [Site key: CFN – Cass flats, CHV – Chilton valley, CVS – Cave stream, DFL – *Dracophyllum* flat lower, DFU– *Dracophyllum* flat upper, LRV – Little river, RCF – Redcliffes station]. a) Viable seed all seasons, b) Viable seed 1998 – 1999, c) Viable seed 1999 – 2000, d) Viable seed 2000 – 2001.



A description of the interpretation of box plots can be seen in Figure 2.1.2.

Table 2.1.4 Analysis of Variance Table, Seed production at field sites 1998–2001 (square root transformed).

Total Seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	6	44.2075	7.367915	8.551005	**0.00000001**
Year	2	12.8382	6.419096	7.449831	**0.0007**
Site: Year	9	17.9559	1.995097	2.315456	**0.02**
Residuals	346	298.1285	0.861643		

Filled Seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	6	161.4738	26.9123	10.28647	**0.00000001**
Year	2	216.7205	108.3603	41.41767	**0.00000001**
Site):Year	9	135.5403	15.0600	5.75628	**0.00000001**
Residuals	325	850.2913	2.6163		

Viable Seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	6	87.922	14.65371	4.23247	**0.0004**
Year	2	77.576	38.78807	11.20327	**0.00002**
Site: Year	9	122.280	13.58671	3.92429	**0.00010**
Residuals	296	1024.814	3.46221		

Frequencies of sexual reproduction

Frequencies of sexual reproduction varied between sites, but all sites produced at least some hybrid progeny. Even sites that were geographically close to each other showed large differences in reproductive mode. This was particularly illustrated at the two *Dracophyllum* flat sites that are approximately 100 m apart. The two sites at Cass, Cass flats and Chilton valley, also varied considerably from each other in the frequency of sexual reproduction recorded over the three years (see Table 2.1.5a-c). Two of the sites examined, *Dracophyllum* flat lower and Redcliffes station, were found to have obligate sexual individuals present. These were recognised by the absence of the maternal type in

the progeny following pollination with *Hieracium aurantiacum*. This can be seen in Appendix 2, with some of the crosses from these sites producing almost all progeny via sex. Following the decapitation of the capitula of these plants (see Figure 1.2.1), no filled seed was produced. If capitula were covered with the field bag apparatus, excluding external pollen sources, again, no viable seed was produced. Pollination with either *H. aurantiacum* or facultative apomictic pentaploid *H. pilosella* produced a high degree of filled seed (Chapman *et al. in prep*). Plants from the *Dracophyllum* flat lower site exhibited more vigour than those from Redcliffes station and also produced more seed following pollination (G. Houliston *pers obs.*). Further investigation has identified that these plants have most likely arisen on site, via a hybridisation event between facultative apomicts (Chapman *et al. in prep*).

Analysis of the role of the environment on residual sexual reproduction at the sites can be seen in chapter IV.1.

Crosses resulting in at least one aberrant individual (putative hybrid) had a significantly greater number of total progeny than those crosses resulting in purely apomicticly derived progeny, when compared with a t-test (averages of 38.14 and 28.04 respectively, t = 4.210, df = 302, p-value = 0.0001).

Table 2.1.5a. Reproductive output of Field Sites, 1998 / 99

Site	Pollinations	Pollinations producing progeny	Mean Progeny per cross*	No. crosses producing hybrids	Total Progeny (all crosses)	No. Sexual† (all crosses)	% **Sexual**
Cave stream	11	11	53.27 ± 0.315	1	586	1	**0.17**
Draco flat lower	8	8	42.00 ± 0.450	3	408	88	**21.57**
Draco flat upper	8	8	51.00 ± 0.310	4	336	5	**1.49**
Cass flats	18	18	32.50 ± 0.597	12	585	35	**5.98**
Chilton valley	24	22	36.09 ± 0.517	4	794	5	**0.63**
Little river	50	49	34.20 ± 0.437	26	1676	45	**2.69**
Redcliffes station	NA	NA	NA	NA	NA	NA	**NA**

*Of those that produced at least one individual †The number of progeny produced via sex

Table 2.1.5b. Reproductive output of field sites, 1999 / 00

Site	Pollinations	Pollinations producing progeny	Mean Progeny per cross*	No. crosses producing hybrids	Total Progeny (all crosses)	No. Sexual† (all crosses)	% Sexual
Cave stream	NA	NA	NA	NA	NA	NA	**NA**
Draco flat lower	13	2	4.00 ± 0.707	2	8	8	**100.00**
Draco flat upper	16	14	31.43 ± 0.793	5	440	8	**1.84**
Cass flats	25	13	11.92 ± 1.143	1	155	1	**0.65**
Chilton valley	28	21	25.48 ± 0.738	4	535	7	**1.31**
Little river	30	30	20.97 ± 0.484	11	629	17	**2.70**
Redcliffes station	8	7	5.86 ± 0.270	6	41	30	**73.17**

*Of those that produced at least one individual †The number of progeny produced via sex

Table 2.1.5c. Reproductive output of field sites, 2000 / 01

Site	Pollinations	Pollinations producing progeny	Mean Progeny per cross*	No. crosses producing hybrids	Total Progeny (all crosses)	No. Sexual† (all crosses)	% Sexual
Cave stream	17	12	22.50 ± 1.134	4	270	4	**1.48**
Draco flat lower	18	8	40.27 ± 0.560	5	152	57	**37.50**
Draco flat upper	25	22	19.00 ± 0.986	7	886	19	**2.14**
Cass flats	19	17	39.76 ± 0.501	10	676	17	**2.72**
Chilton valley	25	17	46.86 ± 0.527	10	984	12	**1.22**
Little river	22	20	27.20 ± 0.605	5	544	10	**1.84**
Redcliffes station	NA	NA	NA	NA	NA	NA	**NA**

*Of those that produced at least one individual †The number of progeny produced via sex

Morphological analysis

In general, putative hybrid progeny could be readily assigned to one of three morphological classes, based primarily on the amount of vigour displayed (Table 2.1.6). These classes are described in more detail in Houliston and Chapman (2001). Putative hybrid progeny were identified from all of the sites examined in all years. Involucral bract hair arrangements were often the most obvious hybrid characteristic prior to flowering of the plants. Leaf morphology in some cases also exhibited considerable hybrid vigour and an intermediate density of stellate hairs on the abaxial surface. Leaf length to width ratio was also often greater than that of either parent. Heterosis was often evident in general plant stature and flower and seed output. Floret colour was often intermediate between the two parents (see Figure 2.1.5). In all cases hybrid progeny were observed to have a darker floret colour than the maternal type, with some approaching pale red. The number of capitula per peduncle was also intermediate between the two parents, although under glasshouse conditions it was common for *Hieracium pilosella* to have two capitula per scape, although these almost always branched towards the base. Hybrid progeny, however, usually had the peduncle branching in the last twenty or thirty millimetres. Putative hybrid individuals also commonly had axial leaves growing from the base of the inflorescence.

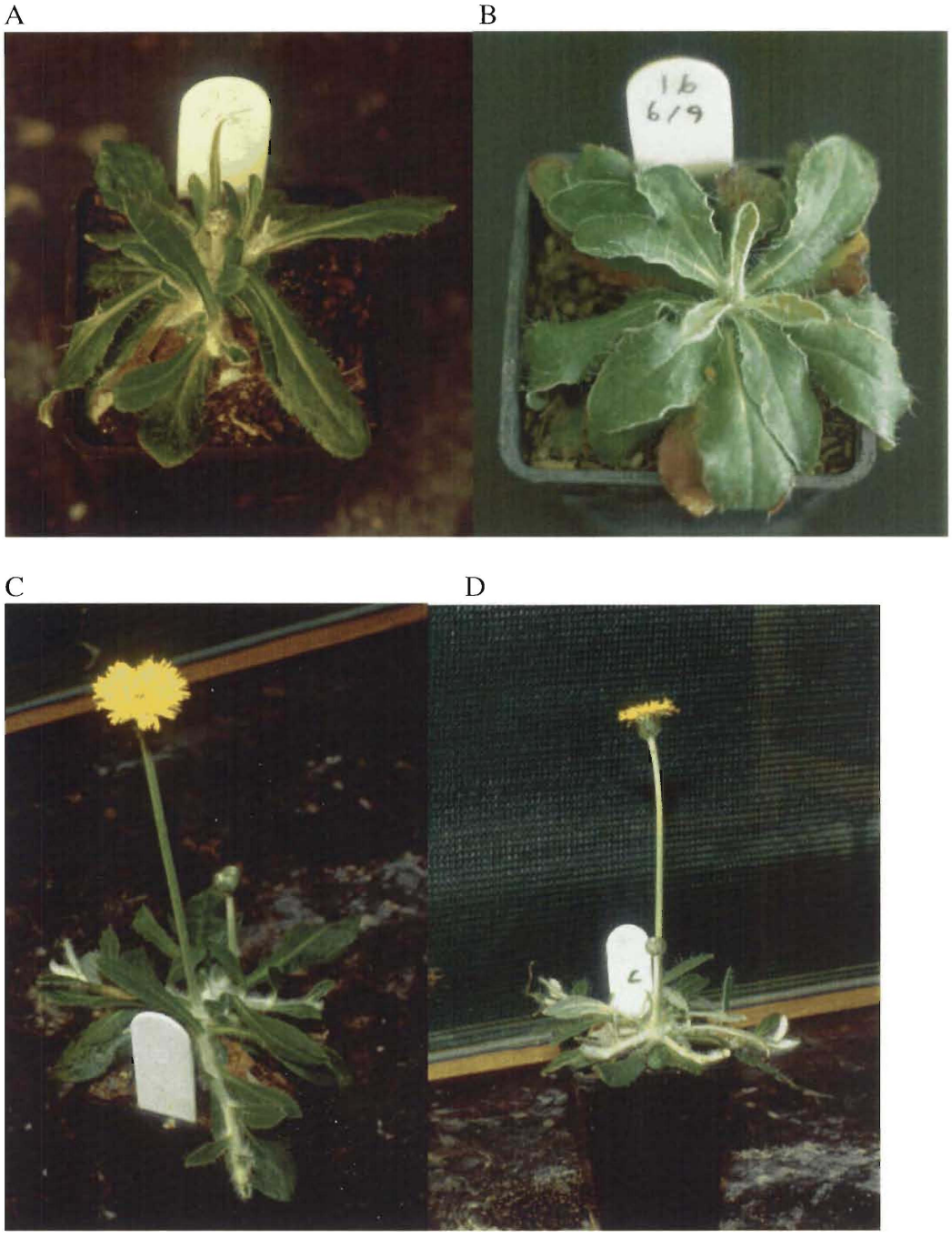
Some aberrant progeny were observed to have small, misshapen leaves, a single capitulum per scape and very low vigour. The leaves also had a dense tomentum of stellate hairs on the underside and were considerably thickened. The glandular involucral bract hairs, however, were more like that of *Hieracium aurantiacum* than *H. pilosella.* That these plants were of hybrid origin was further confirmed using molecular methods (see Chapter II.2.). The occurrence of this morphological type is often observed following artificial pollination of *Hieracium* subgen. *Pilosella* apomicts, and these plants do appear to be the result of amphimixis (R. Bicknell *pers comm.*). In some cases such plants also possessed pale orange ligules, indicating their hybrid origin. Hybrid progeny and their putative parents can be seen in Figure 2.1.5a-b. Examples of the "stunted" morphological class, and the range in hybrid floret colour can be seen in Figures 2.1.6 and 2.1.7 respectively.

Figure 2.1.5a Putative Hybrid 1045b (centre) with Maternal *Hieracium pilosella* (left) and Paternal *H. aurantiacum* (right).

Note the intermediate floret colour and high flowering output of the hybrid (1045b), and the clear morphological differences between the two parents.

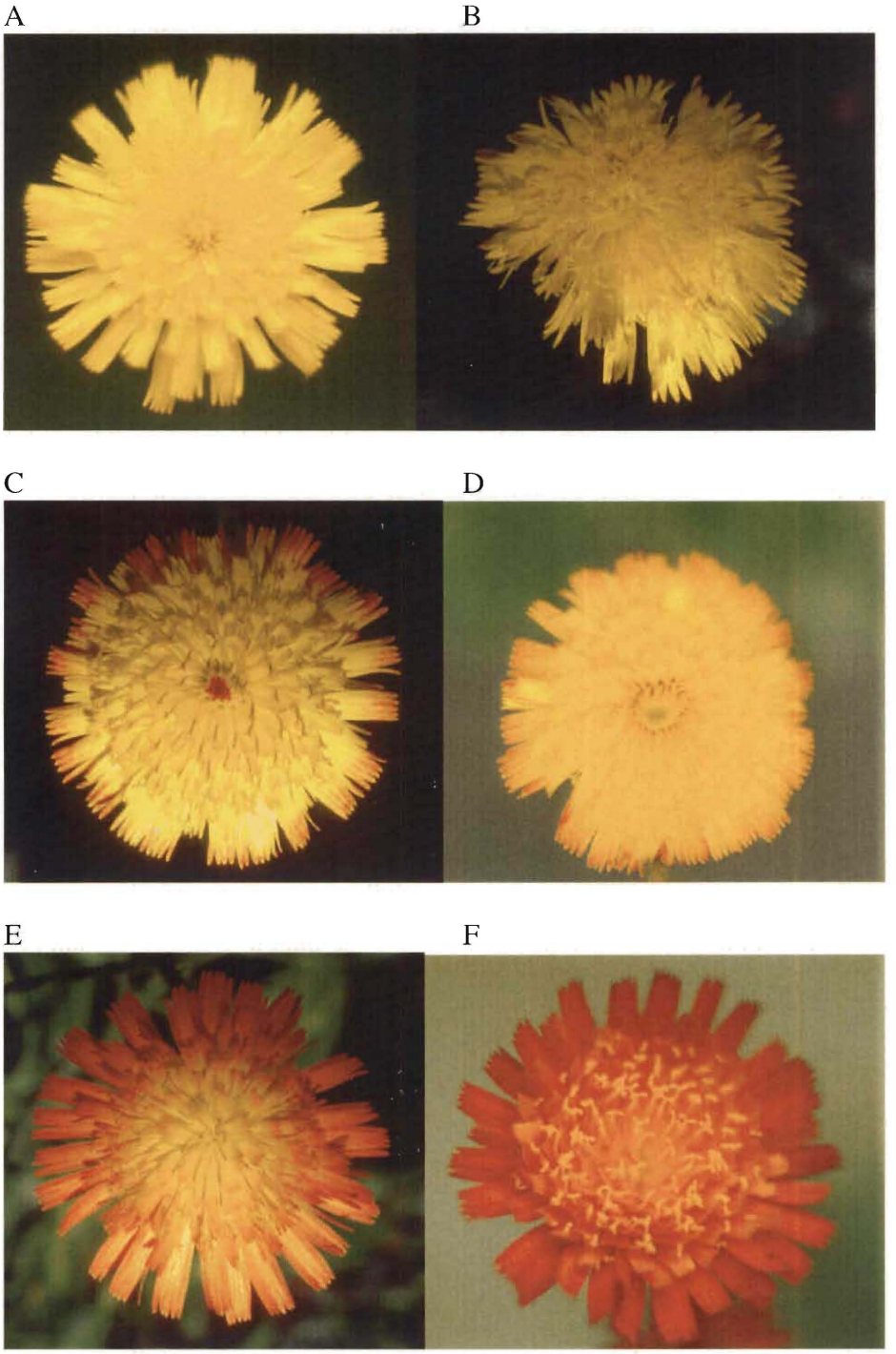
Figure 2.1.5b Putative Hybrid 1003a with Maternal *Hieracium pilosella* (left) and Paternal *H. aurantiacum* (right).

Figure 2.1.6 Hybrids of the "stunted" morphological class.



Key: A: Hybrid 1095a. Note the uneven leaf surface and margins. B: Hybrid 3016a, from the Cave Stream site. Note the fusion of two leaves on the top left corner of the rosette. C: Hybrid 1099b, displaying the abnormal floret development often observed in these plants. D: Hybrid 1096c, typical habit of the "stunted" morphological class.

Figure 2.1.7 Range of floret colour observed in the Hybrid Progeny.



A: Hybrid 1070a, B: Hybrid 1099b ("stunted" type), C: Hybrid 1106b, D: 1106c, E: Hybrid 1014b F: Hybrid 2116c.

Table 2.1.6 Morphological and ploidy characteristics of the parents and F1 hybrids (Subset of 1998 / 1999 season).

All measurements are ± standard error where indicated.

	Maternal parent	Hybrid	Hybrid	Hybrid	Paternal parent
	H. pilosella	"stunted"	"intermediate"	"vigorous"	*H. aurantiacum*
Chromosome No.	36/45	31	27/31/36/42/54	27/ 31/ 36	27/31
Ploidy	4x, 5x	3x+4	3x-6x including aneuploids	3x - 4x, including aneuploids	3x, 3x+4
Capitula characteristics					
Colour	pale yellow	pale yellow	bright orange to dark orange	light orange to dark orange	bright orange
Symmetry	perfect	asymmetrical malformed florets	perfect	perfect	perfect
No. per peduncle	1	1	1 to 4	2 to 8	6 to 18
Rosette Leaves					
Colour	green	glaucous	dark green	dark green	dark green
Shape (Oblanceolate)	narrow	misshapen	broad	broad	narrow-broad
Mean length (mm)	99 ± 0.128	43.79 ± 0.223	91.96 ± 0.105	105.44 ± 0.135	94 ± 0.156
Mean width (mm)	21 ± 0.183	13.36 ± 0.229	27.5 ± 0.148	30.44 ± 0.204	24 ± 0.188
Mean length/ width ratio	5.07 ± 0.089	3.43 ± 0.152	3.59 ± 0.192	4.06 ± 0.232	4.02 ± 0.119
Abaxial surface					
Stellate hairs	many	many/dense	few	few	few to 0
Involucral Bracts					
Mean glandular hair lengths (μm)	500 ± 0.100	644 ± 0.118	593 ± 0.173	585 ± 0.267	676 ± 0.173
Achenes					
Dimensions (mm)					
Length	2.09 ± 0.080	2.16 ± 0.029	2.26 ± 0.143	2.23 ± 0.172	1.82 ± 0.105
Width	0.41 ± 0.138	0.64 ± 0.054	0.45 ± 0.216	0.46 ± 0.137	0.44 ± 0.128
Number and frequency of hybrids in each class		**11 (6.02%)**	**125 (69.92%)**	**43 (24.06%)**	

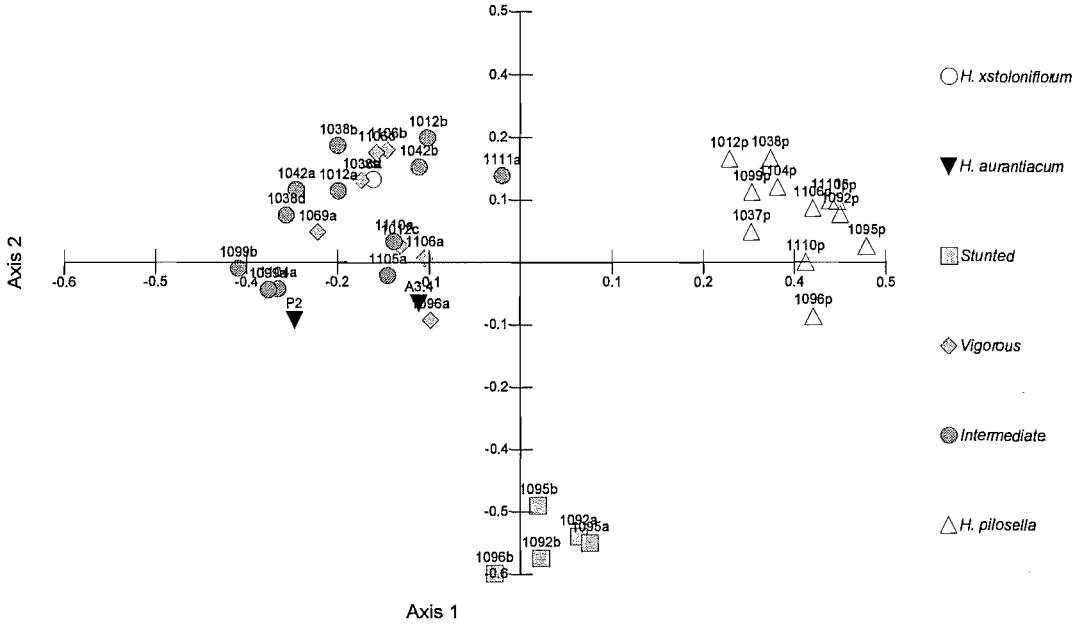
From Houliston & Chapman (2001).

Principle Co-ordinate Analysis of the morphological data resulted in clear distinctions being found between hybrids and both parents. The three axis investigated showed different affinities between hybrids and the parents depending on how they were combined in the two dimensional plot (see Figure 2.1.8a-b). The clustering of these individuals between the two parents indicates the intermediate nature of the morphology of the hybrids. The "stunted" class of hybrid progeny clustered away from all of the other samples on axis 2 (18.33 % of the total variation), although was intermediate between *Hieracium pilosella* and *H. aurantiacum* on axes 1 (24.71 % of the total variation) and 3 (8.86 % of the total variation). Almost all other hybrid progeny fell between the two parents in the two plots with the exception of individual 1096a, which fell below all samples excluding the "stunted" type on axis 2. On both plots 1096a clustered very close to *H. aurantiacum*, and the gross morphology of this plant also resembled that of the paternal parent. *H.* x*stoloniflorum* was found to cluster with the majority of hybrid individuals, particularly those in the "intermediate" and 'vigorous" classes, which is a good indication of the hybrid nature of these individuals.

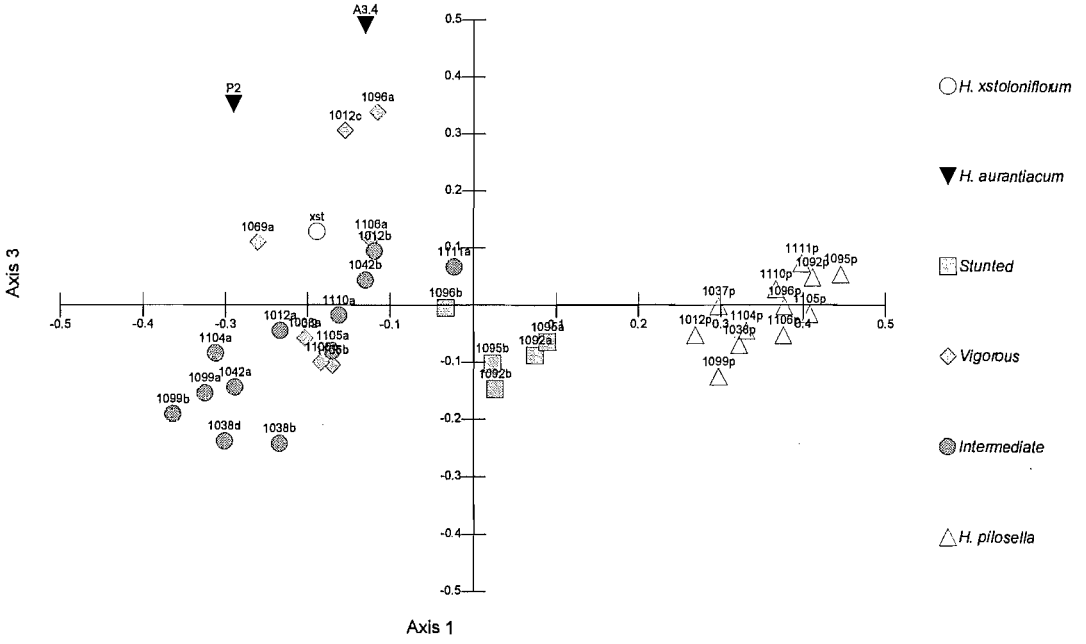
Most putative hybrids were intermediate in gross morphology to the two parents, however individuals with high vigour resembled *Hieracium aurantiacum* to a larger degree and clustered closer to *H. aurantiacum* in the PCA plots. It is unclear whether these vigorous plants were more like *H. aurantiacum* morphologically due to segregation of traits or simply due to an increase in stature (via heterosis), making them more resemble the larger of the two parents. Those plants with less vigour, particularly of the "stunted" morphological class, were morphologically more similar to *H. pilosella* in many characters, especially as they only possessed a single capitula per peduncle.

Figure 2.1.8 Principle co-ordinate analysis of morphological data (Gower general similarity coefficient), hybrid classes and both parents.
Individual code numbers refer to those in Appendix 2 for the field crossing data 1998 /99. Those with a "p" suffix are *H. pilosella*, "a, b, c", refer to hybrid individuals. A3.4 and P2 are the two *H. aurantiacum* accessions used as pollen donors, "xst" refers to *H.* x*stoloniflorum* from the central North Island.

a) Axis 1 & 2.



b) Axis 1 & 3.



The inheritance of apomixis

Hybrid progeny were most often apomictic, although obligate sexual plants on average comprised approximately 20 % of the total hybrid progeny from maternal facultative apomicts (see Table 2.1.7). Hybrids produced from crossing obligate sexual tetraploid *Hieracium pilosella* as the maternal plant produced a ratio of obligate sexual to apomictic progeny of 1:1. This is further evidence for the dominant model of inheritance for apomixis in *Hieracium* spp. As the pollen donor A3.4 is known to have a genotype of Aaa (Bicknell *et al.* 2000), and the obligate sexual tetraploid will be putative aaaa (where A is the dominant gene for apomixis, a the recessive), the expected frequency of obligate sexuals in the hybrid progeny would be 0.50. This is assuming that A3.4 on average produces reduced pollen, and that 50 % of the pollen produced, contains the A allele.

Table 2.1.7 Frequency of hybrids that were obligately sexual from the field crosses, 1999 –2001. Crosses where the maternal parent was also obligately sexual in bold.

Site	1999/00	2000/01
Redcliffes station	0/1 [0.00%] **14/29 [48.28%]**	NA
Cave stream	NA	2/4 [50.00%]
Draco flat lower	0/0 [0.00%] **4/8 [50.00%]**	1/7 [14.29%] **26/51 [50.98%]**
Draco flat upper	1/8 [12.50%]	2/19 [10.53%]
Cass flats	0/1 [0.00%]	7/17 [41.18%]
Chilton valley	2/7 [28.57%]	1/12 [%]
Little river	3/17 [17.65%]	2/10 [20.00%]
TOTAL	6/34 [17.65%] **18/37 [48.65%]**	15/69 [21.74 %] **26/51 [50.98%]**
GRAND TOTAL	21/ 103 [20.39 %] **44/88 [50.00%]**	

Although there was considerable variation in the frequency of obligate sexual plants amongst the hybrids produced from obligate sexual parents, this was considerably less than that observed for the facultative apomictic maternal parents (Table 2.1.7).

II.1.4 DISCUSSION

Seed production and sexual reproduction

Of the 364 putative crosses carried out over the three field seasons, 322 resulted in viable progeny, and 117 resulted in some at least one aberrant individual (hybrid). The populations examined that did not have obligate sexual *Hieracium pilosella* present generally produced between 0.2 – 6.0 % of the total progeny via sex. This shows that the potential for sexual reproduction in predominantly apomictic plants is a widespread phenomenon in this species in New Zealand. Frequencies of sexual reproduction are higher than reported by Chapman and Bicknell (2000) for *H. praealtum* and *H. caespitosum* under glasshouse conditions, although this study has much larger sample sizes. Whether the difference in the ability of these species to act as maternal parents is due to differences in reproductive modes or sampling error is unclear.

The placement of capitula in the pollination exclusion bags did not appear to have any adverse effect on seed production. The scape could still elongate without difficulty, and the nylon mesh did not retain water. No difference was observed under field conditions in seed set from enclosed and open plants (G.J. Houliston *pers. obs.*). The enclosures did prevent external pollen sources reaching the stigmas of the capitulum by excluding insect vectors, although there was some possibility of wind pollination. This is unlikely, however, due to the mesh size of the cloth and the insect pollination syndrome of the capitulum inflorescence.

The amount of seed produced, (total, filled, and viable), varied considerably over the seven sites, with significant differences found between most sites for all three. This indicates that there is potential for both the site, possibly genotype of the plants or an environmental factor without an annual temporal fluctuation, and annual environmental fluctuations to play a role in determining seed output.

The reproductive patterns of *Hieracium pilosella* are complex at the population level. All populations had the capacity for some sexual events, although the majority of the seed produced at all but two of the sites (Redcliffes station & *Dracophyllum* flat lower) is via apomixis. The fact that the majority of seed produced at those sites was via sex is

due to the presence of obligate sexual individuals, rather than high rates of residual sexual reproduction. Although obligate sexual *H. pilosella* has not been previously described in New Zealand (see Chapman *et al. in prep* for the first record), it is interesting to note that it has been found in two of the seven populations examined in this study. Obligate sexual *H. pilosella* has also been discovered at a third site within the Waimakariri basin, between Lake Lyndon and Lake Coleridge. Whether obligate sexual *H. pilosella* is widespread in New Zealand, or is in higher frequencies within the study area is unknown. It would seem unlikely that these represent the entire range of obligate sexual *H. pilosella* in New Zealand, and obviously this question requires further investigation (see Appendix 4). The lack of records for sexual *H. pilosella* in the literature up to the present, and the population structure of these populations, suggest that these plants have arisen on site, most likely following a hybridisation event between facultative apomicts (Chapman *et al. in prep*). The frequency of obligate sexual formation when crossing facultative apomicts is moderate, as can be seen in Table 2.1.7, but it is clear that this mechanism can produce plants with such a reproductive pattern (see below).

The levels of sexual reproduction recorded at the field sites may have been an underestimation for several reasons. All F_1 progeny that were not substantially different to the maternal parent were classed as apomictic. It is possible, but unlikely due to the strong morphological differences between the parents, that some hybrid progeny so resembled the maternal plant that they were assigned to the apomicticly derived progeny. Mentor effects may have allowed the self-fertilisation of the sexual florets in the capitulum, leading to a morphological type resembling the maternal parent, but of sexual origin (Houliston & Chapman 2001). The pollination efficiency of *Hieracium aurantiacum* may be different to *H. pilosella,* and this could lead to an over or under-estimate of the potential for sex. Pollination visitation and the effect of capitulum phenology are discussed below. Although these inaccuracies in measuring the potential for sexual reproduction under field conditions exist, the method used for this study does allow comparisons to be made between populations and environmental conditions. The influence of the environment on the expression of apomixis under field conditions will be addressed in chapter IV.1.

The frequency of abortion of capitula varied among the sites, and particularly between seasons. The 1999 / 2000 field season had by far the greatest abortion rate (see Appendix 3). Climate conditions during this year were different to the other two seasons, with relatively wet conditions in December, followed by almost drought conditions in January and February (see Appendix 5 for details). Abortion of *Hieracium pilosella* capitula is often a direct correlate of water stress (Makepeace 1985b). The tetraploid sexual plants at both the *Dracophyllum* flat lower and Redcliffes station sites appear to possess a higher abortion rate than the pentaploid apomicts. This difference in tolerance to the physical environment may indicate that these plants have greater environmental sensitivity than apomicts. Although this has also been observed in other taxa with both sexual and apomictic types (Bierzychudek 1989, Michaels & Bazzaz 1989), it is difficult to draw strong conclusions due to the limited sample sizes.

The higher number of progeny found in crosses resulting in at least one hybrid individual (where the maternal parent was apomictic) is much greater than would be expected if the difference in reproductive output in the two groups is due to additional offspring following the pollination of reduced embryo sacs. This may indicate that sexual reproduction is favoured under conditions that favour high fecundity, or that individuals with high fecundity also invest more in sexual reproduction.

The lower fecundity of obligate sexual *Hieracium pilosella*, and the high between season variation in this trait shows that there may be a temporal component selecting for sex at the *Dracophyllum* flat lower site. Ceplitis (2001) found that seasonal variation in reproductive output in *Allium vineale* was maintaining a mixed mating system due to different rates of fecundity for seed or bulbil production. The selection pressures maintaining sexual individuals despite their lower reproductive output at these sites are interesting, and it is possible that a similar situation to Ceplitis (2001) exists to maintain these populations. The high potential for seed output in the obligate sexual plants from the *Dracophyllum* flat lower site is evident under glasshouse conditions (see Chapter IV.3), and the adaptation to certain conditions that vary on a temporal scale may allow the coexistence of the two reproductive types.

Morphological analysis

The morphological characteristics of *Hieracium aurantiacum* proved suitable as a marker for sexual reproduction in this subgenus. Both the morphology and difference in chromosome number (see Chapter II.3) allow for easy detection of hybrid origin in the progeny of facultative apomicts (see also Krahulcová & Krahulec 2000).

The three morphological classes of the progeny illustrate the range of morphological types isolated from these crosses. The continuous variation in floret colour of the hybrid individuals illustrated their origin well, as did the intermediate bract hair arrangements. The other characters described in Table 2.1.6 also indicated the hybrid origin of the aberrant progeny. The placing of hybrids into the "intermediate" and "vigorous" classes was largely decided on the number of capitula per scape, the glandular hair length, and the leaf length to width ratio. The influence of ploidy level on the morphological types is further discussed in chapter II.3.

PCO analysis of the morphological data resulted in good resolution between hybrid (aberrant) and apomictic (non-aberrant) progeny on axis 1 & 2 combined and 1 & 3 combined, although in both cases hybrids clustered closer to *Hieracium aurantiacum* than *H. pilosella* (see Figure 2.1.8). Plotting axis 2 & 3 together resulted in the clustering of hybrid and the *H. pilosella* types together. As PCO does not provide resolution to individual characters, it is not possible to say which characters are responsible for the clustering on the three axes plotted. Plotting of further axes was not attempted. The general intermediate position of the hybrid individuals on the two dimensional plots indicates that these plants are intermediate in morphological character, and indeed of hybrid origin. The table of morphological traits (2.1.6) detected in hybrid progeny further indicates this, and it is clear that the hybrid progeny are the result of sexual events following the pollination of *H. pilosella* with *H. aurantiacum*.

Artificial pollination and phenology

Pollinating the capitulum when all florets are fully open leaves potential for meiotic embryo sacs to miss pollination if the age of the stigma is beyond receptivity. Although *Hieracium* spp. possess centrapetalous opening, the variation in development between inner and outer florets is not as pronounced as in other apomictic taxa, such as

Taraxacum or *Calendula* (Rosenberg 1907). A study looking at the rate of sexual reproduction in *Paspalum notatum* concluded that the highest frequency was detectable after pollination at anthesis as opposed to prior to or following this stage (Espinoza *et al.* 2002). Theoretically, pollination at this point in development gives the greatest opportunity for sexual reproduction. The time between anthesis of the capitulum and the closure preceding the beginning of seed set is shorter in New Zealand than in *Hieracium* spp. in Europe. Krahulcová and Krahulec (2000) report pollinating *Hieracium* subgen. *Pilosella* species up to three times in the space of a week in an experimental garden. New Zealand field and glasshouse populations have a window of pollination of between only two to almost three days during summer conditions. Time to seed set in the field in New Zealand is very predictable, usually 18 days after capitulum anthesis for apomicts, 20 days for obligate sexuals (G.J. Houliston *pers obs.*). No reports have been found from Europe on the time between capitula anthesis and seed ripening. Whether this is also truncated in New Zealand is of interest, and may illustrate further differences between the *H. pilosella* group in Europe and New Zealand.

The inheritance of apomixis

The proportion of obligate sexual hybrid progeny produced from facultative apomictic maternal parents for the two seasons recorded was on average 20.39%. As apomixis in *Hieracium pilosella* is controlled by a single dominant allele, the expression of obligate sexuality requires the homozygous recessive state (Bicknell *et al.* 2000). The paternal parent contains one dominant allele for apomixis, two recessive (Aaa). It is unlikely that hybrids of BIII origin will be obligate sexuals as the presence of an unreduced gamete will ensure that at least a single copy of the apomictic allele is present. Therefore, obligate sexual hybrids from facultative apomictic maternal parents will be almost exclusively BII hybrids (see Chapter II.3).

The finding that exactly 50% of the hybrids produced from an obligate sexual tetraploid (putative aaaa) were obligate sexuals when crossed with A3.4 (Aaa), is further evidence for the dominant nature and simple inheritance of apomixis in *Hieracium pilosella* as described by Bicknell *et al.* (2000).

The inheritance of the differential expression of apomixis in facultative apomictic *Hieracium pilosella* has not been addressed by this study. Recent works have found that modifiers to the primary apomixis locus may influence the expression of the developmental pathway coded for by the apomixis gene (Koltunow *et al.* 2000, Bicknell *et al.* 2001). The complexity of the inheritance of facultative apomixis combined with the potential environmental control of this trait make a study of this untenable within the boundaries of this project.

Pollinator visitation

Potential insect vectors were often observed visiting *Hieracium pilosella* inflorescences. Insect visitors to *H. pilosella* were most commonly Native bees (Hymenoptera, Apoidae), although bush flies (Diptera, Muscoidae) and hover flies (Diptera, Syrphoidae) were also frequently observed. A preliminary study of the visitation rates of pollen vectors on *H. pilosella* found that the rate was greater than that of *Celmisia gracilenta,* a native daisy species included for comparison (Dean Scott & H.M. Chapman *unpubl. report*). As apomictic *H. pilosella* produces comparable pollen to the amphimictic type (see Chapter II.4) pollen feeding insects will receive a reward by visiting *H. pilosella* inflorescences, and therefore potential for pollination of facultative apomicts exists.

Further evidence that at least some pollination is effected is the presence of interspecific hybrids at the *Dracophyllum* flat lower site, and an absence of completely empty, non-aborted capitula at the sites with sexual plants present (G. J. Houliston *pers obs.*). The presence of pollination vectors is imperative if the potential for sexual reproduction in *Hieracium pilosella* is to be met under field conditions. Production of viable pollen in *H. pilosella* will be addressed in chapter II.4.

II.2.1. RANDOM AMPLIFIED POLYMORPHIC DNAS AND INTER-SIMPLE SEQUENCE REPEATS AS A CONFIRMATION METHOD FOR HYBRID PROGENY OF *HIERACIUM PILOSELLA*.

Molecular methods to detect hybrid origin

Molecular methods are commonly applied to studies of breeding system in numerous organisms. They provide the advantages of speed, accuracy and wide applicability (Weising *et al.* 1995). This study has primarily used morphology (see Chapter II.2) to determine the hybrid origin of aberrant plants in the progeny of facultative apomicts, and this also is a suitable method, particularly in cases with high phenotypic variability and low plasticity. Molecular methods will be used for a subset of the putative hybrid progeny to confirm the origin of these plants. The intermediate morphological traits found in the hybrid progeny do indicate a hybrid origin, however molecular methods have the advantage of supplying random, unbiased markers for such analysis. Some progeny also displayed morphology that was more indicative of one of the two parents; molecular techniques can be used to confirm whether such individuals are indeed hybrids. Confirming the hybrid origin of some of the aberrant progeny using molecular methods will give an indication of the suitability of using morphology to identify hybrids in this study.

Molecular methods, particularly DNA fingerprinting techniques, have revolutionised both reproductive and population biology. There are many different DNA based techniques for the detection of genetic diversity at the population and individual level. Most techniques employ the Polymerase Chain Reaction (PCR) to amplify specific regions of the genome using oligonucleotides and a thermally stable DNA polymerase, before the fragments are separated by electrophoresis to provide polymorphic markers to determine differences in genotype (Weising *et al.* 1995).

RAPD and ISSR techniques

In this study, two PCR based techniques are used to attempt to confirm the hybrid origin of aberrant individuals. Both RAPDs (Random Amplified Polymorphic DNAs) and ISSRs (Inter – Simple Sequence Repeats) share the characteristics of simplicity, speed, and high levels of positive results with primers (Weising *et al.* 1995). These techniques

are chosen in preference over AFLPs (Amplified Fragment Length Polymorphisms) due to the simplicity of their preparation, and the fact that the products can be separated and visualised on agar gels. Other techniques, such as RFLPs (Restriction Fragment Length Polymorphisms) and sequencing, provide much more resolution than is required in a study such as this. Initially it was intended to use ISSRs for this study, but following methodological difficulties; they were substituted with RAPDs.

RAPDs and ISSRs both use arbitrarily chosen oligonucleotide sequences (primers) to select areas of the genome for amplification. RAPD primers are 10 bases long, typically have a GC content between 50-80%, and avoid palindromic sequences (Williams *et al.* 1990). ISSRs use simple base repeats [with or without a two or three base pair "anchor" on the 3' or 5'end], resulting in a primer 17 to 22 bases long (Charters *et al.* 1996, Fang *et al.* 1998). ISSR primers do not have a base content prescribed, and often contain palindromic sequences; some are even a complete palindrome. These are used to amplify DNA sequences bordered by these priming sites, before fragments are separated and resolved by staining with a DNA intercalating stain. Both methods result in dominant markers (Williams *et al.* 1990, Ziętkiewicz *et al.* 1994). RAPDs, and to a lesser extent ISSRs, have been criticised for lack of repeatability, but recent works have shown this is not the issue it once was. Some of the problems have been due to these methods being employed for the analysis of small or degraded DNA samples (Weising *et al.* 1995). When screening plant genomic DNA using fresh tissue this is not such a concern, especially with *Hieracium* spp. where DNA extraction is simple and results in high yields (G.J. Houliston *pers. obs.*). If the yield is low, or of poor quality, then contamination from external sources can result in erratic results (Weising *et al.* 1995), although much smaller amounts of template is required than for RFLPs (Charters *et al.* 1996). Weising *et al.* (1995) found that while these methods are repeatable by the same operator, using the same equipment, there might be some variation in the banding patterns found between different laboratories. Moreno *et al.* (1998) examined the effects of different equipment and laboratories on RAPD profiles and found that although different banding patterns were recovered from different laboratories, the same conclusions were drawn, particularly if weak bands were discarded from the analysis. The main source of variation in the results of these techniques was attributed to the slightly different thermal profiles of the various thermocyclers (see also Weising *et al.* 1995, Huff & Bara 1993).

It is predicted that ISSRs will be more robust than RAPDs due to their longer primer lengths and higher annealing temperatures, but this has not always been reflected in comparisons between the two techniques (Hollingsworth *et al.* 1998). Sweeny *et al.* (1996) reported that no variation between replicates of extractions had been found when using RAPDs to characterise strains of a turf grass, and they are now regarded to be reasonably robust in comparison to other PCR techniques. A study using repeats of 5'-anchored SSR reactions (ISSR with the addition of a three base pair "anchor" on the 5' end) found no variation between replicates, indicating the repeatability of this method (Charters *et al.* 1996).

ISSRs rely on primers that contain repeat sequences of bases, as would be expected to be found in non-functional areas of the genome. These regions have been described as being "transcriptionally silent". This is an advantage when using an area of the genome as a marker, as meiotic mutation rates have been quoted as being up to 5%, resulting in high marker polymorphism in populations (Weising *et al.* 1995). These areas also comprise a large proportion of the genome, providing numerous variable loci and therefore many potential markers (Charters *et al.* 1996).

Detecting interspecific hybrids is a common application of the RAPD method. Arnold *et al.* (1991) used only three RAPD primers to confirm the hybrid origin of *Iris nelsonii,* when compared with the putative parents *I. fulva* and *I. hexagona*. Even the origins of intergeneric hybrids have been studied using RAPDs. Thirteen primers were used to identify species specific bands in *Margyicarpus digynus* and *Acaena argenta,* and the profiles of these species compared to the putative hybrid *xMargyricarpus skottsbergii.* This analysis discounted *A. ovalifolia* as a putative parent based on evidence from RAPD profiles (Crawford *et al.* 1993).

RAPDs have also been employed in studies of apomictic taxa, to identify aberrant progeny. It was declared "an efficient screening tool for discriminating maternal plants and recognising the hybrid constitution of aberrant plants" in the aposporous apomict, *Poa pratensis* (Barcaccia *et al.* 1997).

ISSRs have been used in many studies where the detection of variation between closely related individuals has been required. A study looking at clonal grapevine varieties found that even very closely related lines could be determined using ISSRs, and that the repeatability of this technique was very good, ranging between 86 and 94% for the resulting banding patterns. It is interesting to note that strains resolved using ISSRs could not be determined by RAPDs (Moreno *et al.* 1998). Ziętkiewicz *et al.* (1994) also reported ISSRs detected more polymorphisms per reaction than RAPDs. Other works have found that the two methods detect a comparable amount of polymorphism (see Hollingsworth *et al.* 1998).

ISSRs have been previously used in a population study of *Hieracium pilosella* (as *Pilosella officinarum*) in New Zealand, and considerable variation has been detected within this single species (Chapman *et al.* 2000). The fact that such variation was discovered within a species would indicate that this technique may be suitable to characterise interspecific hybrids between *H. pilosella* and *H. aurantiacum.*

II.2.2 MATERIALS AND METHODS

DNA extraction

Extraction of DNA was carried out on a sub sample of the progeny isolated from the field crosses during 1998 / 1999 (see Chapter II.1). Samples screened from a single capitulum of *Hieracium pilosella,* crossed with *H. aurantiacum* under field conditions, included both morphologically aberrant (putative hybrid) and non-aberrant individuals. As demonstrated in chapter II.1, it is clear the non-aberrant individuals are of the *H. pilosella* type, and therefore most likely the product of apomixis. By comparing profiles of these two groups of progeny from the same capitulum, it is possible to further confirm the origin of hybrids. DNA was also extracted from *H. aurantiacum*, the putative paternal parent [both A3.4 and P2 accessions] to allow detection of markers from this species in the hybrids, further confirming hybrid origin. Confirming the hybrid origin of morphologically aberrant progeny with molecular markers will determine if intermediate

morphology can be used with confidence to detect hybridisation, and therefore sex, in *H. pilosella.*

DNA isolation was carried out using a modified CTAB procedure (Doyle & Doyle 1987). Fresh leaf tissue was ground in a mortar containing 1.0ml of CTAB extraction buffer (0.40 ml dH20, 0.1 ml 1M Tris, 80 µl 0.25M EDTA, 0.087 gm NaCl, 0.40 ml 5M CTAB, 20 µl β-mercaptoethanol). The resulting slurry was transferred to a 1.5ml eppendorf tube and incubated for 30 minutes at 65° C. 750 µl of SEVAG (24:1 Chloroform / IsoAmyl Alcohol) was added, before inverting the tubes to mix, and centrifuging for 4 minutes at 12000 rpm. The clear upper phase was pipetted into a clean eppendorf tube and 750 µl of cold isopropanol added. The solution was left for 24 hours at -20°C for the DNA to precipitate, and centrifuged for 4 minutes at 12000 rpm to produce a pellet. The isopropanol was decanted off and 1 ml of 70% ethanol added before repeating the centrifugation. The pellet was then air-dried for approximately 2 hours, and resuspended in 100 µl of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). Resuspended DNA was separated on a 1% agarose (Seakem LE) gel with 1x Tris-Acetate buffer (0.08 M Tris-phosphate, 0.002 EDTA), after which the gel was stained with 1mg/l Ethidium Bromide. Lambda quantity markers were used to determine the amount of DNA recovered from each reaction, and each was diluted to 20 ng before being used for either ISSR or RAPD PCR reactions. This improves the repeatability and resolution of the results from either of the two techniques, and is especially critical for RAPDs (A. Catanach *pers comm*).

RAPD methodology

Random Amplified Polymorphic DNAs (RAPDs) were performed following a modification of the procedure used by A. Catanach at Crop and Food Research Limited, Lincoln.

RAPD PCR was performed as a 25 µl reaction per sample:

Volume (µl)	Constituents
2.5	10x Taq polymerase PCR buffer (Boehringer Mannheim)
1.5	25 mM Magnesium Chloride (Boehringer Mannheim)
0.25	10 mM dNTPS (Boehringer Mannheim)
0.5	20 µM Primer
0.2	Taq DNA polymerase (Boehringer Mannheim)
20.55	dH20
1.0	suspended DNA product
25.0	

Primers were custom ordered from Gibco BRL based on the Operon J kit (see Table 2.2.1). All reactions were covered by 10 µl of mineral oil to prevent evaporation.

Table 2.2.1 RAPD Primers (Gibco BRL), Operon J kit.

Primer Number	Primer Sequence (5′- 3′)
Operon J-06	TCG TTC CGC A
Operon J-09	TGA GCC TCA C
Operon J-10	AAG CCC GAG G
Operon J-11	ACT CCT GCG A

The reaction was amplified in a PTC-200 Thermal Cycler (MJ Research). Initial denaturation was 60 seconds at 92°C, followed by cycles of 92°C for 60 seconds, 35°C for 60 seconds, and 72°C for 120 seconds, for a total of 41 repeats. Final extension was 72°C for 6 minutes, and the reaction halted by a constant 4°C treatment.

ISSR methodology

Inter simple sequence repeats (ISSRs) were performed as a modification of the procedure used by Chapman *et al.* (2000).

ISSR PCR was performed as a 25 µl reaction per sample:

Volume (µl)	Constituents
2.5	10x Taq polymerase PCR buffer (Boehringer Mannheim)
1.25	10 mM dNTPS (Boehringer Mannheim)
6.0	25 µM Magnesium Chloride (Boehringer Mannheim)
1.0	10 mM Primer
0.25	Taq DNA polymerase (Boehringer Mannheim)
13.0	dH20
1.0	suspended DNA product.
25.0	

Primers used were University of British Columbia primer set 9, (see Table 2.2.2).

Table 2.2.2 University of British Columbia Primer Set #9.

Primer Number	Primer Sequence (5′- 3′)*
UBC 900	ACT TCC CCA CAG GTT AAC ACA
UBC 845	CTC TCT CTC TCT CTC TRG
UBC 822	TCT CTC TCT CTC TCT CA

*R= purine

The reaction was amplified in a PTC-200 Thermal Cycler (MJ Research). Initial denaturation was 4 minutes at 93°C, followed by cycles of 93°C for 20 seconds, 52°C for 60 seconds and 72°C for 20 seconds, for a total of 41 repeats. Final extension was 72°C for 4 minutes and the reaction was then halted by a constant 4°C treatment.

Agarose gel electrophoresis

PCR products were stored at -20°C before being separated on a 2% Tris Acetate gel (3:1 NuSieve agarose). The gel was then stained for 30 minutes in 1mg/l Ethidium Bromide, destained for 2 minutes in water, before being viewed under UV light. Images were collected using a Kodak electrophoresis digital camera, and colour was inverted using Adobe PhotoShop (Version 5.0). Banding patterns were scored for the four primers used,

and the matrix entered into an excel worksheet for collation prior to analysis. Only bands that were clear and repeatable were included in the analysis.

Data analysis

Banding patterns were imported into Multi Variate Statistical Program [MVSP version 3.1a] (Kovach Computing Services 1999), and analysed using Principle Co-Ordinate Analysis (PCO) with a Jaccard's similarity measure (details of Jaccard's similarity can be seen in Table 2.2.3). A total of three eigenvectors were projected in a two-dimensional scatter plots, and the individuals labelled either as *H. pilosella, H. aurantiacum,* or hybrid. As there were more bands than individuals, PCO was the most appropriate method for these data (see Chapter II.1.2).

Jaccard's similarity was used in all cases as this method only takes into account positive matches between bands, and as missing bands in RAPD profiles can be for several different reasons, this is one of the most appropriate methods (Weising *et al.* 1995).

Table 2.2.3 Jaccard's Coefficient.
Jaccard's coefficient (similarity measure) is defined as (modified from MVSP Version 3.1a, Kovach Computing Services (1999):

$$S_J = Nsp / (Nsp + Nu)$$

Where: Nsp is the number of bands shared by the two individuals,
Nu is the number of bands present in one of the individuals, but not the other, in the pair-wise comparison.

The repeatability of RAPDs was determined by comparing the banding patterns for *Hieracium aurantiacum* present on each gel. Reactions were also repeated and run alongside each other on a single gel for comparison (see Figure 2.2.3).

II.2.3 RESULTS

ISSRs

ISSRs produced banding patterns with all primers that were screened. It was possible to determine the difference between *Hieracium pilosella* and both *H. aurantiacum* and *H. praealtum,* but it was not possible to detect polymorphisms within *H. pilosella,* or within aberrant progeny. Most primers produced many bands, often of similar molecular weight, which made the discrimination between individuals impossible. Separation of products on acrylamide did not improve the resolution of the banding patterns.

RAPDs

RAPD primers were selected due to their known ability to discriminate between closely related individuals of the subgenus *Pilosella* (A. Catanach *pers comm.*), and four particularly useful primers were found to detect differences between both parents and aberrant progeny. All primers produced a high proportion of polymorphic bands (see Table 2.2.3). The proportion of polymorphic bands for *Hieracium pilosella* alone was less than for all the samples, indicating that markers other than those from *H. pilosella* were present in the profiles.

Of the total 32 bands detected, 7 were found in *Hieracium pilosella* and the hybrids but not *H. aurantiacum*; 6 found in *H. aurantiacum,* four of which were also present in hybrids, but not in *H. pilosella*; and three were unique to hybrid individuals. The remaining 16 were found in both *H. pilosella* and *H. aurantiacum,* and were therefore of little use when assigning putative parentage. An additional two bands were present in *H. aurantiacum* and some hybrids, and also in single individuals of *H. pilosella.* The two accessions of *H. aurantiacum* used in this study differed by the absence of two bands in A3.4 that were present in the P2 accession. These two bands also occurred in some hybrid individuals, and additionally, both in *H. pilosella* sample 1095p, and one band in *H. pilosella* sample 1069p.

Table 2.2.3. Numbers of Polymorphic bands per RAPD Primer.

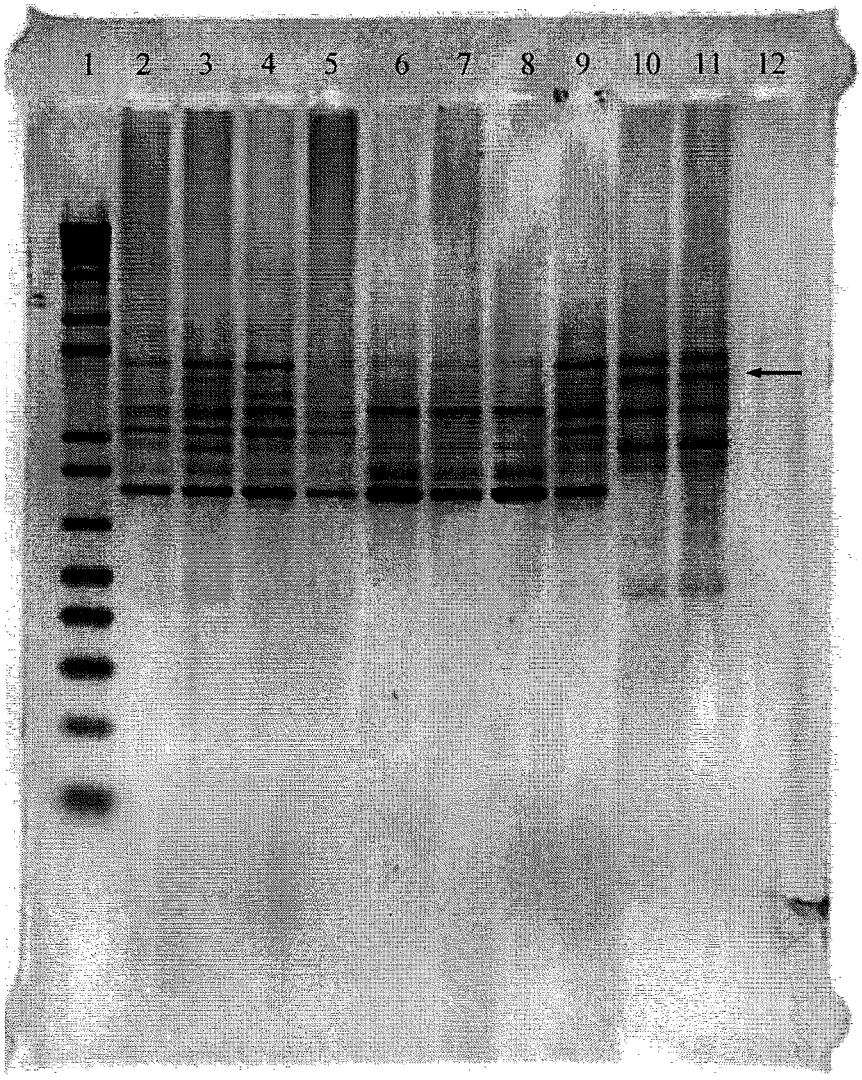
Primer	Number of bands per primer	Number of polymorphic bands (*H. pilosella* only in parentheses)
Operon J-06	10	10 (6)
Operon J-09	12	10 (5)
Operon J-10	6	5 (4)
Operon J-11	4	4 (1)

Examples of gel images for scoring can be seen in Figures 2.2.1-2.2.2. Figure 2.2.3 shows reactions repeated in pairs. It is clear that RAPDs are a repeatable method, provided the same methodology is followed for each sample. Digital photographs have had the colour inverted to improve resolution of the bands. As can be seen in Figure 2.2.1, banding pattern differences between maternal parents and hybrids were not always discernible with a single primer. All hybrid individuals screened, however, were shown to be different from the maternal parent with the combination of primers used. Profiles of hybrids were often much more like those of the maternal parent, *Hieracium pilosella,* than the paternal *H. aurantiacum.* With all primers there were clear, repeatable, differences between *H. pilosella* and *H. aurantiacum.*

Principle co-ordinate analysis

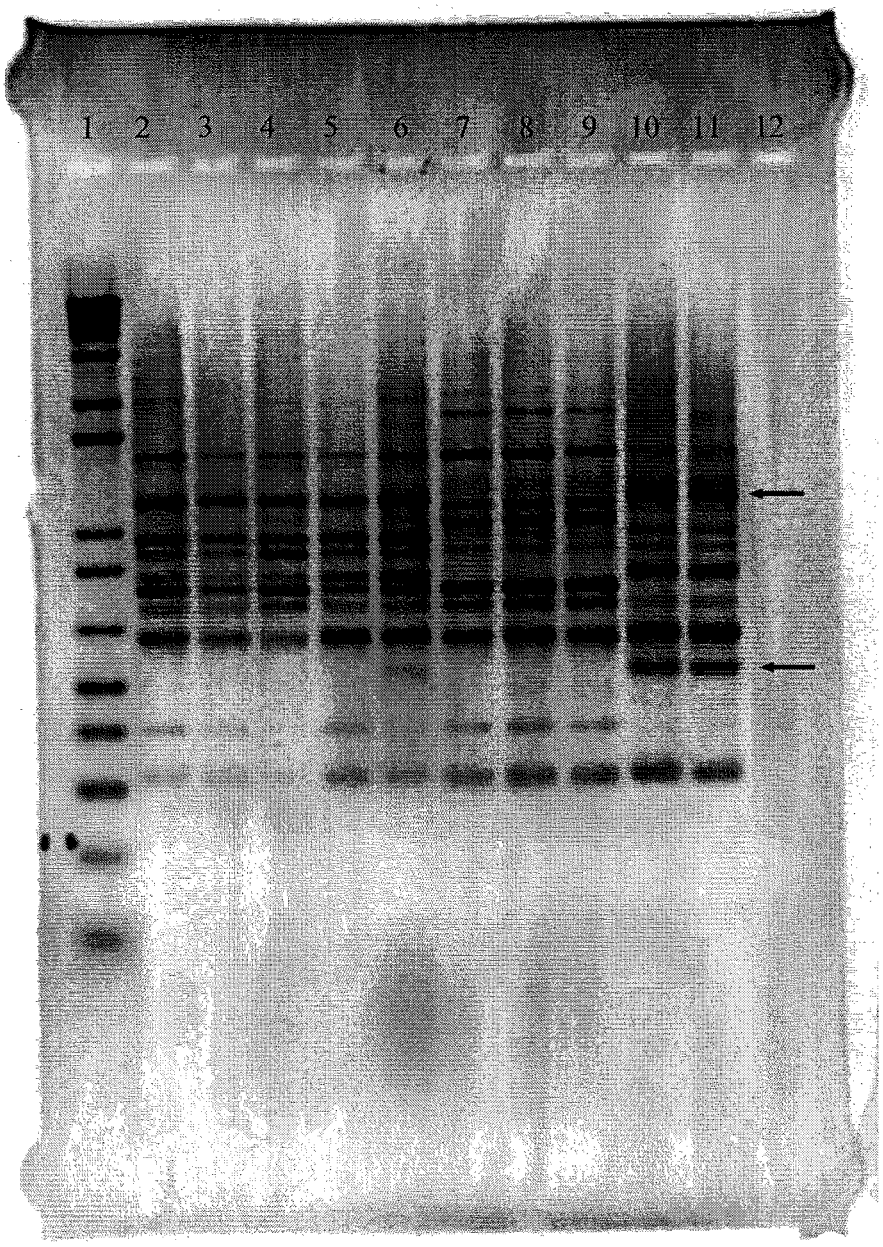
Figure 2.2.4a-b displays the scatter plots of the PCO. *Hieracium aurantiacum* is clearly different from both *H. pilosella* and the hybrid offspring. It can also be seen that all of the hybrid individuals share greater similarity with *H. pilosella* (the maternal parent) than *H. aurantiacum.* Most separation of the parental and hybrid individuals was contained on axis 1 of the PCO, explaining 16.24 % of the variation. Both axes 2 (11.83 % of the variation) and 3 (10.00 % of the variation) did not strongly discriminate between the parents and hybrids. This reflects the low number of bands specific to *H. aurantiacum.* However, the fact that all hybrid individuals are positioned in the plots between their two parents, indicates this is a useful method for determining hybrid origin.

Figure 2.2.1 RAPD Reaction, Putative Hybrids and Parents, Primer J06.



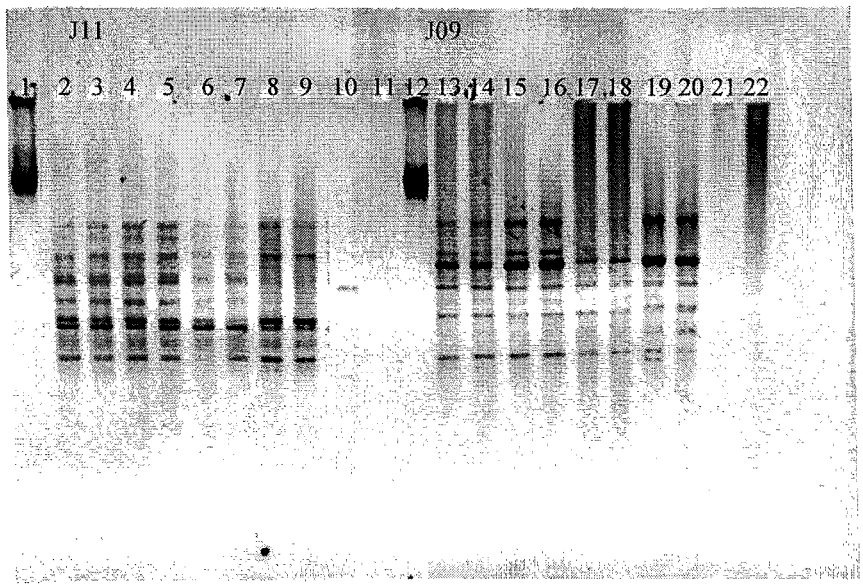
Key; LANES: 1- 1 kb ladder, 2- 1069 *H. pilosella*, 3- 1069 Hybrid (a), 4- 1093 *H. pilosella*, 5- 1093 Hybrid (a), 6- 1093 Hybrid (b), 7- 1095 *H. pilosella*, 8- 1095 Hybrid (a), 9- 1095 Hybrid (b), 10- *H. aurantiacum (*P2), 11- *H. aurantiacum* (A3.4), 12- Negative control. Arrow indicates band specific to *H. aurantiacum* also observed in some hybrid individuals.

Figure 2.2.2 RAPD Reaction, Putative Hybrids and Parents, Primer J10.



Key; LANES: 1- 1 kb ladder, 2- 1069 *H. pilosella*, 3- 1069 Hybrid (a), 4- 1093 *H. pilosella*, 5- 1093 Hybrid (a), 6- 1093 Hybrid (b), 7- 1095 *H. pilosella*, 8- 1095 Hybrid (a), 9- 1095 Hybrid (b), 10- *H. aurantiacum* (P2), 11- *H. aurantiacum* (A3.4), 12- Negative control. Arrows indicate bands specific to *H. aurantiacum* also observed in some hybrid individuals.

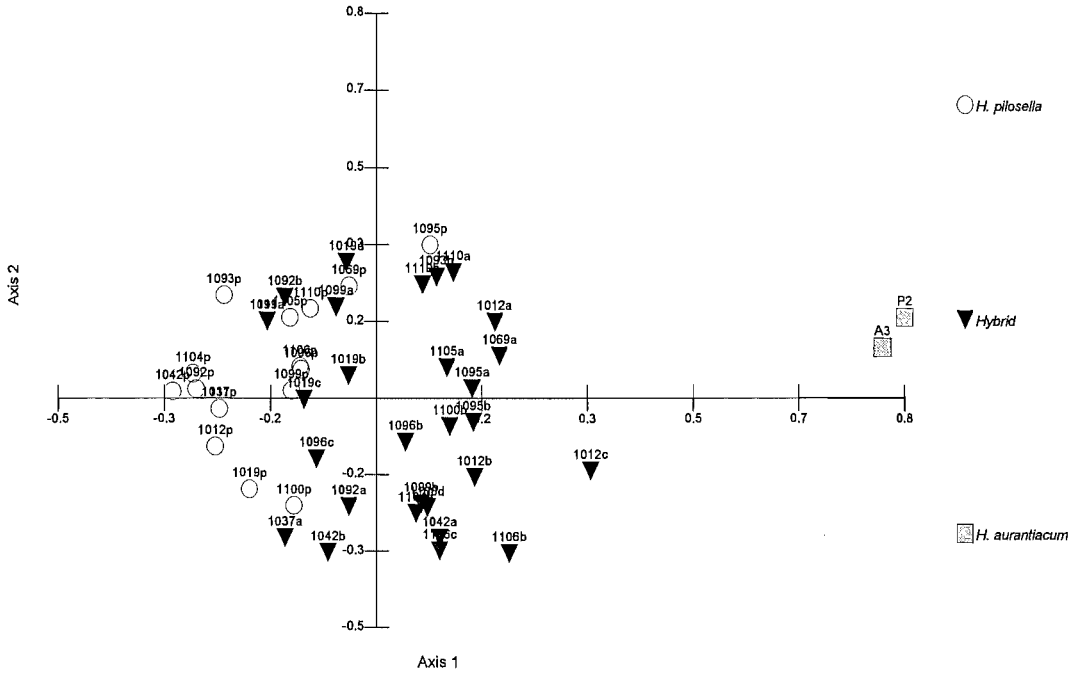
Figure 2.2.3 RAPD Reaction, Repeats of DNA samples with Primers J09 and J11.



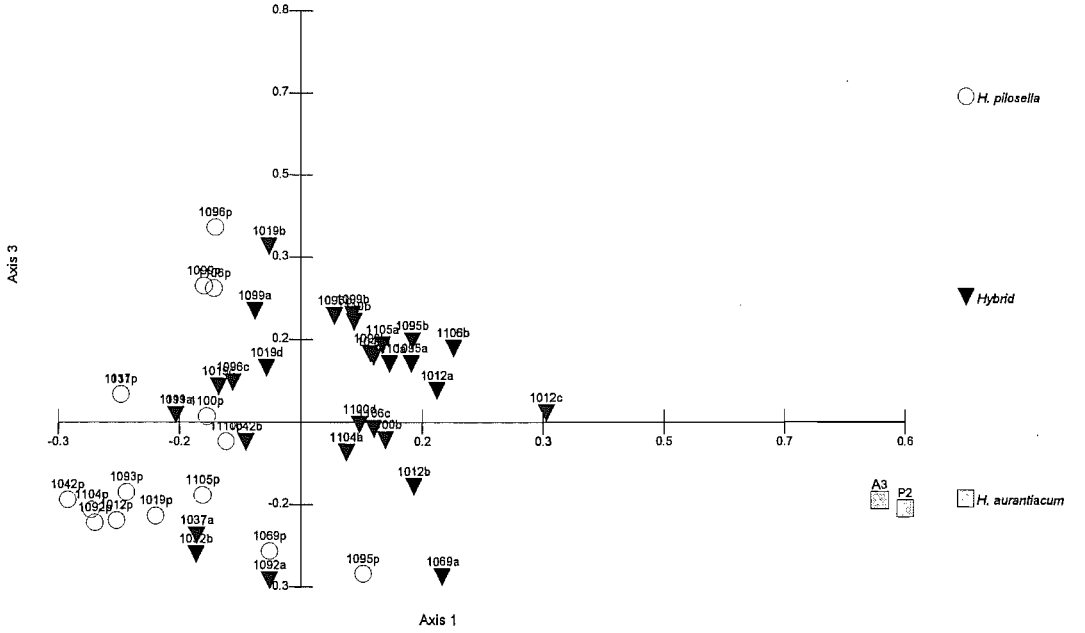
Key; Lanes: 1- 1 kb ladder (degraded), 2 & 3- 1092 *H. pilosella,* 4 & 5- 1099 *H. pilosella,* 6 & 7- 1100 *H. pilosella,* 8 & 9- 1106 *H. pilosella,* 10 & 11- Negative controls J11. 12- 1 kb ladder (degraded), 13& 14- 1092 *H. pilosella,* 15 & 16- 1099 *H. pilosella,* 17 & 18-1100 *H. pilosella,* 19 & 20- 1106 *H. pilosella* 21 & 22- Negative controls J09.

Figure 2.2.4. PCO case scores, RAPD binary data with Jaccard's distance measure. Individual code numbers refer to those in Appendix 2 for the field crossing data 1998 /99. Those with a "p" suffix are *H. pilosella*, "a, b, c", refer to hybrid individuals. A3 and P2 are the two *H. aurantiacum* accessions used as pollen donors.

2.2.4a) Axis 1 & 2.



2.2.4b) Axis 1 & 3.



II.2.4 DISCUSSION

RAPDs performed considerably better than ISSRs for the determination of hybrid origin in aberrant progeny. The failure of ISSRs to distinguish hybrids and parents was due to methodological problems (see Chapter III for an alternative protocol) making it impossible to compare the two techniques. The high number of bands amplified using the ISSR technique led to profiles that were difficult to separate, even using acrylamide. Bands were less distinct than with RAPDs, and only determination at the interspecific level was possible. Electrophoresis using less product per lane may have been necessary, or reduction of the $MgCl^2$ concentration to give more specific amplification (see Chapter III).

RAPD fingerprinting techniques were found to be simple method for delimiting hybrids. The crosses between *Hieracium pilosella* and *H. aurantiacum* at the field sites have produced individuals that are intermediate between the two parents in respect to RAPD banding pattern, and can therefore definitively be declared hybrids. The combination of RAPDs and PCO did not produce a graphical representation of the individuals examined that placed hybrid individuals in an intermediate position in all cases, particularly on axis 2 & 3. This may have been due to the low number of primers used. It is possible to see from the scatter plots, however, that putative hybrids are mostly intermediate between *H. pilosella* and *H. aurantiacum.*

RAPD analysis was found to be repeatable, and no differences could be detected within an individual extraction from a sample. This indicates the method is robust, which is reflected in the analysis of the banding patterns for parents and hybrids.

Banding patterns from hybrids were more like those of *Hieracium pilosella* than *H. aurantiacum.* The fact that *H. aurantiacum* was triploid or hyper-triploid may have influenced this. If the gametes from one of the parents are generally of lower ploidy than the other, then this parent will contribute less to the genome of the offspring. It is

interesting to note that this is the opposite finding to the morphological study (Chapter II.1), where the offspring shared more characters with the paternal parent. This may be due to heterosis effects in morphology, as *H. aurantiacum* generally has larger structures than *H. pilosella,* making it difficult to detect hybrid, versus heterosis, expression. Another factor potentially influencing this was the selection of primers that are known to detect high levels of polymorphism in *H. pilosella* (A. Catanach *pers comm.*). These primers may not be quite as effective for the *H. aurantiacum* genome, and this may explain some of the bias in banding patterns. Conversely, the high proportion of hybrid progeny that were of BIII origin, due to the absence of reduction division during the formation of the female gamete (see Chapter III), may have increased the maternal contribution to the genome of the hybrids.

It is clear that the origin of aberrant progeny is most likely due to hybridisation events. Both the genetic and morphological data indicate *Hieracium aurantiacum* has been included in the parentage, making the use of a morphologically distinct pollen donor and hybrid characteristics for detecting sexual events appropriate for this study. This has also shown that the use of simple molecular techniques to determine parentage between closely related individuals is possible with a limited number of primers.

Although molecular methods were found to be useful for identifying hybrid individuals, morphological methods were found to be more time, and cost, effective. It is clear, however, that molecular methods are more appropriate in situations where morphological differences between parents are not so easily defined.

II.3.1 CYTOLOGICAL INVESTIGATION OF *HIERACIUM PILOSELLA* AND HYBRID PROGENY FROM *H. PILOSELLA* X *H. AURANTIACUM* CROSSES.

Ploidy variation in *Hieracium pilosella* has been studied in many different works. This investigation concentrates on ploidy level as a method to confirm the hybrid origin of aberrant progeny, and to confirm the ability of apomictic *H. pilosella* with different ploidy levels to produce offspring via facultative sex. The first is a relatively simple task, as it is possible to compare the ploidy level of the aberrant and non-aberrant progeny. If the ploidy levels are different it is a good indication that the aberrant progeny are indeed hybrids. The other potential use of this technique is that by examining ploidy level it should be possible to determine if the hybrid was formed from a reduced or unreduced megaspore mother cell (meiocyte). This will allow the definitions BII (reduced) and BIII (unreduced) hybrid to be assigned to the progeny.

Ploidy level and reproductive mode

Ploidy levels in Europe are considered to be good predictors of reproductive mode, with tetraploids assumed to be almost always sexual, and pentaploids apomictic (Gadella 1972, 1987, 1991a, 1991b). The origin of high ploidy levels (up to octoploid), in *H. pilosella* has also been of interest to evolutionary biologists. A high ploidy level such as those detected by Skalińska (1971, 1973) in *H. aurantiacum* (up to octoploid) is indicative of a taxon that can possess extreme environmental tolerance (Bierzychudek 1987a). Ploidy level in New Zealand has mostly been studied as an indicator of population structure and breeding system. Earlier works (Makepeace 1981, Jenkins & Jong 1997, Jenkins 1995) have concluded that the low variation in ploidy level and the prevalence of pentaploids indicates obligate apomictic populations, and clonal, stoloniferous, reproduction. A less emphasised factor was the potential high physiological tolerance of these individuals. The potential for these individuals to possess multiple alleles at each locus means there is a good chance that one allele will code for a beneficial trait under a particular environmental regime (Neuffer & Eschner 1995). This is especially true if the ploidy event has followed hybridisation, generating high internal heterozygosity, as is thought to occur in the origin of many apomicts (Bierzychudek 1989).

Gadella (1987) stated that because only pentaploids were found when crossing a pentaploid maternal line with a tetraploid paternal line, no sexual reproduction had occurred. However, during meiosis in *Hieracium* subgen. *Pilosella,* gametes with different levels of reduction are formed (Bicknell *et al.* 2000), and potentially a sexual progenitor with the same chromosome number as the maternal parent could arise. Although the use of pollen donors with relatively low ploidy levels in this study (triploid [2n = 3x = 27] and hyper-triploid [2n = 3x + 4 = 31]), minimise the chance of this, chromosome number will not be used as a definitive test of hybrid origin.

Manual investigation of ploidy level

Conventional methodology of chromosome counting relies predominantly on the preparation of root tip tissue. Suspension of cell division at late metaphase, followed by the staining of chromosomes, allows the determination of ploidy level using light microscopy. The advantage of this technique is that it is possible to determine actual chromosome number, regardless of relative DNA content of the individual chromosomes. In some cases it is also possible to identify individual chromosomes, and the morphology of the karyotype has been of use in some studies of breeding system (see Krahulcová *et al.* 2001). The disadvantages are that it is time consuming, requires tissue in perfect condition, and can be difficult to perform accurately without extensive training.

Flow cytometry

Flow cytometry (FCM) is a relatively new technique to plant science, having originally been developed for medical applications. A flow cytometer analyses optical properties of stained cells passing through a point of measurement, where they are excited by a light source, most commonly from an ultra-violet lamp or argon laser. Cells are separated as they pass the optical sensor (point of measurement) by a constant stream of sheath fluid, which hydrodynamically constrains the cells to a narrow path, and provides a steady stream of cells for measurement. A conventional objective lens focuses the resulting fluorescence of the cells, which passes through a series of filters to an optical sensor. The signal is then converted to an electrical pulse, and the result recorded on a histogram via computer (Doležel 1997). Preparation of cell suspensions for analysis is both quick and simple, and highly accurate results are possible with little optimisation.

The most common use of FCM in plant science is the estimation of nuclear DNA content of cells (see Cavallini *et al.* 1993, Doležel & Göhde 1995, Pfosser *et al.* 1995, Johnston *et al.* 1999). FCM is ideal for this as it measures cells individually, and can process large numbers of cells in a very short time. Although other methods, such as biochemical assays, absorption microspectrophotometry, cytofluorometry and image analysis exist, FCM has been declared the most accurate and efficient method for the quantification of nuclear DNA content.

An aside to the actual quantification of the DNA content of cells is the comparison of relative amounts of DNA in different cell lines. An obvious application of this is determination of ploidy level. By comparing the position of the peak from an unknown individual on a histogram with that from a conspecific individual with a known ploidy level, it is possible to accurately estimate the ploidy level of the unknown individual. It is for this reason that FCM is so useful for population studies such as this work. When parents differ considerably in ploidy level it is possible to detect interspecific hybrids, and whether progeny are the result of a BII or BIII hybridisation event (Doležel 1997).

This study has selected DAPI (4', 6'-diamidino-2-phenylindole) as the stain for all flow cytometry. The reasons for choosing DAPI are that it is possible to use an ultra-violet lamp for excitation, and the combination of the two provides excellent results, minimising the coefficient of variation on the peaks of the histograms (Doležel & Göhde 1995). DAPI is also non-toxic and preparation of samples is simpler than with other methods. The disadvantage of DAPI is that it has an AT base preference. This makes it unsuitable for quantification of total genomic DNA, but this is not a concern for ploidy measurement providing the same internal standard is used each time. The most common alternative to DAPI is propidium iodide (PI). This stain requires a longer wavelength for excitation, usually from an argon laser, and is highly toxic. Although this is an intercalating stain, it often results in higher CVs than DAPI. Due to the fact that PI also binds to double-stranded RNA, it is necessary to include a ribonuclease in the staining solution. PI is commonly employed for determination of actual genomic DNA content and is superior for this task (Doležel 1997). The advantages of DAPI, however, make it the more suitable method for ploidy determination.

II.3.2 MATERIALS AND METHODS

Chromosome counts

Two methods for chromosome counts were used. The first method (aceto-orcein) was replaced with the second (lacto-propionic orcein) at the end of the first year of research as it offers better resolution and simplified preparation.

Aceto-orcein staining

Fresh root material was collected from sand under potted plants. The roots were placed in an eppendorf tube with distilled water and left in ice at 4°C for 20 hours. This prevents mitosis by inhibiting spindle polymerisation between prophase and anaphase. The root material was placed in a saturated solution of para-dichlorobenzene and 0.3 mg/ml (0.002 M) 8-hydroxyquinoline, placed in a shaker bath, and shaken vigorously for two hours. This collects metaphase figures and condenses the chromosomes.

The roots were then transferred to fresh eppendorf tubes and 1 M HCL was added, before incubation for 15 minutes at 60°C. This softens the cell walls and makes slide preparation easier. The root meristem was trimmed off, leaving only the last 2 mms, and flooded with aceto-orcein stain (45 mls of 45 % acetic acid, 1 g of synthetic orcein, 55 mls of distilled water). The slide was then heated until vapour was just visible, and left to stain for two hours. The excess stain was then washed off with 45 % acetic acid, and a cover slip added. The tissue was squashed and viewed under an oil immersion lens. Squashing was achieved by gently tapping down a coverslip with a disposable pipette tip, and finally by finger pressure under blotting paper (S. C. Lambie *pers comm.*). Only cells with an intact cytoplasm and well-separated chromosomes were used to determine ploidy level.

Lacto-propionic orcein staining

Fresh root material was collected as per the previous method. The roots were placed in an eppendorf tube containing a saturated solution of α-bromonapthalene and left at room temperature for three hours. This condenses chromosomes and halts cell division. Root

tips were then transferred to Farmers' solution (3:1 ethanol-acetic acid) for fixation and left at room temperature overnight.

Root tips were transferred to fresh eppendorf tubes containing 1 M HCL, before incubation for 7 minutes at 60°C to soften the cell wall and make slide preparation easier. The root tips were trimmed, leaving only the last 2mms, and flooded with lacto-propionic orcein stain [2 g synthetic orcein, 50 ml lactic acid, 50 ml propionic acid, dilute to 45 % with distilled water before use] (Jong 1997). The tissue was instantly squashed as per the previous method and viewed under an oil immersion lens (Krahulcová & Krahulec 1999). It was also sometimes necessary to lift an edge of the coverslip with a razor blade to ensure penetration of the stain (A. Krahulcová *pers comm.*). Only cells with an intact cytoplasm and well separated chromosomes were used to determine ploidy level.

Sampling

A sub sample of the 1998 / 1999 field crosses had ploidy recorded for both some hybrid and apomicticly derived progeny (codes of the samples recorded can be seen in Table 2.3.1). The examination of apomicticly derived progeny allowed the chromosome number of the maternal parent to be determined, as both will possess the same ploidy level. Both paternal lineages, P2 and A3.4, also had their chromosome number confirmed using these methods.

Flow cytometry

Equal quantities of leaf tissue of each type to be analysed were placed in a petri dish, to a total area of 24 mm^2. 0.5 mls of Partec UV CyStain precise T solution A (100 ml deionised water, 2.1 g citric acid, 0.5 g Tween 20) (Partec GmbH, Münster, Federal Republic of Germany) was added, and the tissue chopped finely with a stainless steel razor blade. Total extraction time was approximately 90 seconds. The sample was then filtered through a 30 μm filter, and 2.0 mls of Partec UV Cystain Precise T solution B (100 mls deionised water, 7.9 g dibasic sodium phosphate, 0.5 ml DAPI stock [4.55 mg 4', 6'-diamidino-2-phenylindole, 10 ml deionised water]) was added. Samples were analysed after at least 90 seconds of staining for optimal results. In all cases at least 2000 and more frequently 5000 cells were used to determine the ploidy level of the samples, and each measurement was recorded twice.

A Partec PA-II Particle Analysing System (PAS) was used in all counts. Excitation was with a high-pressure mercury arc lamp (HBO 100 W/2, Osram, Federal Republic of Germany) producing ultra violet light. The light source for excitation was passed through a UG 1 filter and a TK420 beam splitter before reaching the flow cell to ensure integrity of the wavelength. The wavelength for excitation was 360nm, with emission at 460nm (Doležel 1997). Signals were passed through a TK 590 long-pass dichoric beam splitter, and finally past a GG 435 long-pass filter before reaching the blue photo multiplier tube (PMT). Analysis was carried out using FloMax version 2.3 (Partec 2001).

Instrument settings varied depending on the material used, but at all times were adjusted to maximise the separation of peaks on the histogram, whilst minimising the lower level noise due to small particle interference.

The internal standards used in all cases were either a sexual tetraploid line of *Hieracium pilosella* collected from the *Dracophyllum* flat lower site, *Bellis perennis* collected from the University of Canterbury lawns, or *Bromus* sp. (Gramineae) from the University glasshouses. *B. perennis* was found to be the best standard for *H. pilosella*, with a nuclear DNA content approximately half that of a tetraploid *H. pilosella*. This species also gives sharp peaks with very little noise signal. These were to provide an internal reference for determining the peak position for each measurement. At no time was external standardisation employed in this study due to the potential inaccuracies this can cause (Greilhuber 1998). Samples were run in random order.

Sampling

A single example of an apomictic progenitor from each of the field crosses during the 1999 / 2000 and 2000 / 2001 seasons had the relative nuclear DNA content determined using FCM. Plants that produced only hybrid progeny (obligate sexuals) were found to be tetraploid following further investigation of ramets collected from these sites (*Dracophyllum* flat lower and Redcliffes station).

Hybrid progeny from each of the three morphological categories described in chapter II.1 were measured for nuclear DNA content and scored as either BII or BIII (addition)

hybrids. For these examples both parents were also included in the sample to see if the hybrid peak fell between the parents, or further right on the histogram. Those individuals that produced clear peaks to the right of the parents were declared BIII hybrids, others as BII.

A random sample of hybrids from the 2000 / 2001 season also had the nature of hybridisation determined using FCM. Representative samples from the six sites were chosen, and the nuclear DNA content compared to the two parents using *Bellis perennis* as an internal standard. Individuals with a DNA ploidy equivalent value of 2n = 48.00 or greater were recorded as BIII hybrids, as this is approximately the maximum value for pentaploid plants from any of the six sites, and therefore any plant with a higher level must be an addition hybrid. Included in this sample were hybrids resulting from crosses between obligate sexual *Hieracium pilosella* and A3.4.

II.3.3 RESULTS

Chromosome counts

Metaphase figures from *Hieracium pilosella* could be discerned using the described methods. Root tip material had to be in excellent condition to yield useful results. The frequencies of each ploidy level can be seen in Table 2.3.1. No aneuploid individuals of *H. pilosella* were found amongst the maternal parents. Hybrids were found to differ in ploidy level to the parents, and in most cases ploidy was intermediate between the two. Because the paternal parent was substantially lower in ploidy than the maternal type, this method was found to be useful for the confirmation of hybrid origin. Due to the relatively low sample sizes of each of the two maternal ploidy levels, it is not possible to draw conclusions on any difference in the ability of each type to produce progeny sexually. Frequencies for the paternal parent were determined from the frequency of two accessions used in the putative crosses, not from direct counts (2n = 3x = 27 – accession P2; 2n = 3x+4 = 31 – accession A3.4).

Table 2.3.1 Frequency of chromosome numbers from a subset of the parents (1998 / 99 field season), and their F_1 hybrid progeny (percentages in parentheses). Determined by direct counts of root tip material.

	2n = 3x = 27	2n = 3x+4 = 31	2n = 4x = 36	2n = 4x+6 = 42	2n = 5x = 45	2n = 6x = 54
H. aurantiacum (paternal parent)	91 (50.8)	88 (49.2)	0 (0)	0 (0)	0 (0)	0 (0)
H. pilosella (maternal parent)	0 (0)	0 (0)	10 † (36.8)	0 (0)	18 * (63.2)	0 (0)
Hybrids ‡	8 (34.8)	5 (21.7)	6 (26.1)	3 (13.0)	0 (0)	1 (4.4)

From Houliston & Chapman (2001).

† 4x maternal parents: 1012p, 1018p, 1037p, 1069p, 1104p, 1105p, 1106p, 1100p, 1111p, 1113p.

* 5x maternal parents: 1005p, 1014p, 1017p, 1024p, 1039p, 1042p, 1045p, 1064p, 1092p, 1093p, 1094p, 1095p, 1099p, 1103p, 1105p, 1108p, 1110p, 1112p.

‡ Hybrids: 2n = 27: 1012b, 1012c, 1019b, 1038a, 1099a, 1099b, 1104a, 1110a.
2n = 31: 1042a, 1069a, 1092a, 1095a, 1096a.
2n = 36: 1019a, 1037a, 1038c, 1104a, 1105a, 1111a.
2n = 42: 1106a, 1106b, 1106c.
2n = 54: 1038b.

Flow cytometry

Resolution using UV excitation of the DAPI fluorochrome was sufficient to determine ploidy level of *Hieracium pilosella* when DNA content was compared to either tetraploid *H. pilosella*, or an external standard. Coefficients of variation for the histogram peaks ranged from 1.2 to 4.0 %, and were typically around 2 % (see Figure 2.3.1).

Ploidy of maternal parents from the field

Frequencies of ploidy level for the maternal parents can be seen in Table 2.3.2. Most sites were comprised mainly of pentaploid individuals, with tetraploids present at the Little river and Chilton valley sites. The Redcliffes station and *Dracophyllum* flat lower sites were predominantly tetraploid, although these sites are mostly obligate sexual individuals.

The proportion of crosses that resulted in at least one hybrid progenitor with tetraploid plants (facultative apomictic and obligate sexual combined), was higher than in pentaploids (facultative apomictic) (Table 2.3.2). However, when the proportion of facultative apomicts with hybrid progenitors is compared, pentaploids were more likely to have produced at least one hybrid (33% of crosses compared to 25% for tetraploid

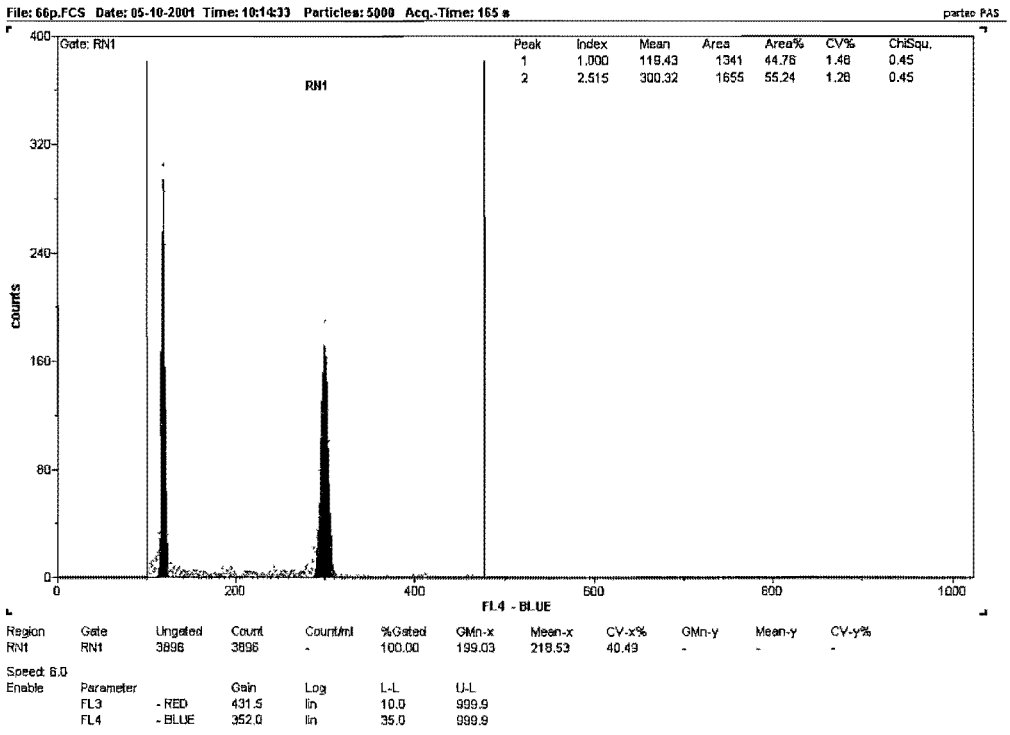
apomicts), although sample sizes for tetraploids were relatively low (Table 2.3.2). Due to the relatively small sample sizes, meaningful statistical analysis was not possible.

Table 2.3.2 Frequencies of ploidy levels at field sites as determined by FCM (percentage in parentheses), 1999-2001.

Site	Year	No. 4x	No. 4x with hybrids	No. 5x	No. 5x with hybrids
Redcliffes station	1999 / 00	7 (100.00)	6 (85.71)	0 (0.00)	0 (0.00)
	2000 / 01	N /A	N / A	N / A	N / A
Cave stream	1999 / 00	N / A	N / A	N / A	N / A
	2000 / 01	0 (0.00)	0 (0.00)	12 (100.00)	4 (33.33)
Cass flats	1999 / 00	0 (0.00)	0 (0.00)	12 (100.00)	1 (8.33)
	2000 / 01	0 (0.00)	0 (0.00)	17 (100.00)	10 (58.82)
Chilton valley	1999 / 00	4 (22.22)	1 (25.00)	14 (77.78)	3 (21.43)
	2000 / 01	0 (0.00)	0 (0.00)	21 (100.00)	8 (38.10)
Dracophyllum flat upper	1999 / 00	0 (0.00)	0 (0.00)	12 (100.00)	1 (8.33)
	2000 / 01	0 (0.00)	0 (0.00)	22 (100.00)	7 (31.81)
Dracophyllum flat lower	1999 / 00	2 (100.00)	2 (100.00)	0 (0.00)	0 (0.00)
	2000 / 01	3* (37.50)	3 (100.00)	5 (62.50)	2 (40.00)
Little river	1999 / 00	4 (14.29)	1 (25.00)	24 (85.71)	10 (41.67)
	2000 / 01	0 (0.00)	0 (0.00)	20 (100.00)	5 (25.00)
TOTAL		**20 (11.43)**	**13 (65.00)**	**155 (88.57)**	**51 (32.90)**
Apomicts only TOTAL		**8 (4.91)**	**2 (25.00)**	**155 (95.09)**	**51 (32.90)**

*including one individual with a DNA ploidy equivalent of 40.2.

Figure 2.3.1 Flow Cytometry histogram of pentaploid *Hieracium pilosella* and diploid *Bellis perennis* (standard) – UV excitation of the DAPI fluorochrome.



Peak index is used to calculate the relative DNA ploidy of the sample based on a standard value for a known tetraploid plant. The calculated value per chromosome from the standard accession was 0.054, therefore the DNA ploidy of this sample (2066p) was 46.23 (peak index / calculated value per chromosome).

DNA ploidy equivalents of hybrid progeny

The random sample of hybrids (not based on morphological class) produced from both facultative apomictic and obligate sexual parents produced a wide range of ploidy levels (see Table 2.3.3). Variation in DNA ploidy equivalents in the hybrid individuals was high, ranging from 39.0 to 98.0 chromosome equivalents of *Hieracium pilosella*. The average DNA ploidy equivalent of all the hybrids measured was 56.2, with a standard deviation of 17.12, demonstrating the high ploidy levels often found in the hybrid progeny. The proportion of BII to BIII hybrids produced from facultative apomictic *H. pilosella* was not significantly different to 1:1 ($p = 0.866$, Chi-square test, $df = 1$). No BIII hybrids were detected in the hybrid progeny of the tetraploid sexual plants.

Table 2.3.3 Frequency of BII and BIII hybrids produced by facultative apomicts and obligate sexuals, sub-sample of the 2000 / 2001 season.

Maternal Parent	Facultative Apomictic		Obligate Sexual	
Hybrid type	BII	BIII	BII	BIII
% of total hybrids examined	17 / 35 = 48.57 %	18 / 35 = 51.42 %	9 / 9 = 100.00%	0 / 9 = 0.00%
Sample codes	3014a-b, 3034d, 3036a-b, 3059a-b, 3086a-b, 3086b-c, 3088a, 3090a, 3092a, 3116a, 3148a, 3152b	3034a-b-c, 3055a-b, 3055b, 3058a, 3065a, 3074a-b, 3092a, 3118a-b, 3120a-b, 3121a, 3145a, 3148b, 3152a	3022a-b-c-d-e, 3037a-b, 3038a-b	

There was no relationship between BII or BIII hybrid origin and the placing of individuals into the morphological classes "intermediate" and "vigorous" (see Chapter II.1), although the "vigorous" class had a higher proportion of BII hybrids. The "stunted" class, however, was entirely comprised of BIII hybrids, based on DNA ploidy equivalents (Table 2.3.4).

Table 2.3.4 Hybridisation type frequencies, by differing morphological type.

Morphological type	BII hybrids (% in parentheses)	BIII hybrids (% in parentheses)
"Stunted"	0 (0.00%)	4 (100.00%)
"Intermediate"	3 (50.00%)	3 (50.00%)
"Vigorous"	5 (83.33%)	1 (16.67%)

Among population nuclear DNA variation

A survey of variation of pentaploid plants from the seven sites found significant differences in DNA ploidy equivalents among populations (Tables 2.3.5, 2.3.6). A summary of population differentiation can be seen in Table 2.3.7. The amount of within-site variation also differed, with some sites possessing more variation in nuclear DNA content than others (Table 2.3.5).

Table 2.3.5 DNA Ploidy equivalents, average 5x value per site (± Std. Err.) 1999 / 00 & 2000 / 01 seasons.

Site	DNA Ploidy equivalents	No. of Samples
Redcliffes station*	46.912 ± 0.0146	7
Cave stream	46.801 ± 0.0069	12
Dracophyllum flat lower	46.620 ± 0.0210	6
Dracophyllum flat upper	46.629 ± 0.0127	36
Cass flats	45.331 ± 0.0098	29
Chilton valley	47.236 ± 0.0150	36
Little river	45.669 ± 0.0173	45
TOTAL	**46.304 ± 0.0209**	**171**

* 5x value from Chapman *et al.* (*in prep*).

Table 2.3.6 Single Factor ANOVA of DNA ploidy equivalents of pentaploids, for the seven populations sampled.

Analysis of Variance Table.

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Site	6	84.14380	14.02397	31.04975	**0.00001**
Residuals	163	73.62078	0.45166		

Table 2.3.7 Least significant difference test of mean DNA ploidy equivalents, pentaploids only.
Sites that share a line beneath them are not significantly different at $\alpha = 0.05$ (solid line), ($\alpha = 0.01$ (dashed line)).

Cass flats	Little river	*Draco* flat L	*Draco* flat U	Cave stream	Redcliffes	Chilton valley
_____	_____					
		_____	_____	_____		
----------	----------				_____	_____
		----------	----------	----------		
				----------	----------	----------

There was no geographic concordance between variation in nuclear DNA content and site, nor was there a relationship with the frequency of residual sexual reproduction (data not shown).

II.3.4 DISCUSSION

Facultative sex and ploidy level

The ability of facultative apomictic *Hieracium pilosella* to reproduce sexually does not seem to be influenced substantially by ploidy level. Although sample sizes for tetraploid plants were low, there appeared to be no difference between the level of facultative sexuality in tetraploid and pentaploid apomicts. The presence of tetraploid apomicts in a population therefore, is unlikely to be a useful indicator for higher than average levels of residual sexuality. However, as all sexual plants recovered from the field to date are tetraploid, FCM may provide a fast way to initially survey populations for the presence of potentially obligate sexual plants.

Population level ploidy variation

The majority of *Hieracium pilosella* at the sites examined had DNA ploidy equivalent levels indicative of pentaploids. Using flow cytometry, it was not possible to be certain about instances of aneuploidy unless there was a large change in chromosome number. Number 3037 was an aberrant individual with a DNA ploidy equivalent indicative of 40

chromosomes (2n = 4x + 4 = 40). That this was found at the *Dracophyllum* flat lower site is not surprising, with several instances of aneuploidy in this population (Chapman *et al. in prep*). Aneuploids are expected to be more numerous at the sites with obligate sexuals present due to the higher rates of sexual reproduction, and pentaploid individuals at these sites acting as paternal parents, producing pollen with uneven ploidy. Although this is possible at sites with facultative apomicts, the probability is much lower as even pentaploid plants often produce diploid gametes (Gadella 1987). A3.4 is a useful marker in this respect as we expect a high proportion of pollen formed by this plant to be aneuploid and therefore any hybrids between this and *H. pilosella* also have a high chance of being aneuploid.

Ploidy levels of hybrid progeny

Ploidy levels of the hybrids were interesting for the high proportion of BIII hybrids present, not different to 50 % in the progeny of facultative apomicts. Due to the relatively low samples sizes it is difficult to know if this is due to sampling or a genetic trait of meiosis in *Hieracium pilosella.* No BIII hybrids were found among the progeny of crosses between obligate sexual tetraploids and A3.4, indicating that the majority of viable pollen produced by A3.4 is reduced, either haploid or diploid; making this a useful pollen donor for detecting BIII events in *H. pilosella.* That BIII events are found in facultative apomictic *H. pilosella* shows that female meiosis is irregular, and that there is a high occurrence of unreduced, sexual, egg cells. The ploidy level of pollen was not detectable using flow cytometry and conventional nuclei extraction methods (hypotonic solution and mechanical pressure) due to the high levels of noise, possibly due to background fluorescence of pollen wall constituents. Sample 3086 is noteworthy as the four hybrids from this sample all were of the BII type. This is contrasted by samples such as 3034 where three are BIII, with one BII present. This may indicate that some facultative apomictic *H. pilosella* genotypes have more regular meiosis than others, but it is unclear whether this is a genetic trait, or is under environmental influence. Bergström (1969) found that in tetraploid apomictic *Hieracium* subgen. *Hieracium robustum,* meiosis in pollen was more regular at higher temperatures, indicating that perhaps some environmental influence is possible.

A comparison of nuclear DNA content variation in *Hieracium pilosella* with other published values

The level of nuclear DNA content variation in and between the field populations of *Hieracium pilosella* shows that populations have some level of genetic differentiation. A 4% difference in nuclear DNA content was found between the highest and lowest values from the seven populations, and significant differences in pentaploid nuclear DNA content were found between most sites. The high significance of the differences observed show that this is due to population level changes in DNA or base content, rather than error in the measurements. It is unclear what the potential mechanism for these disparities is, but there are several possibilities. As DAPI is a non-intercalating stain with an AT preference (Doležel *et al.* 1992) there is some potential for changes in base frequency leading to differences in the estimate of nuclear DNA content level. Whether such differences are sufficient to explain the level of variation observed is unclear, and this level of variation is certainly greater than other authors have found when using DAPI as a fluorochrome for FCM (see Table 2.3.8.). The level of variation between *H. pilosella* populations even approaches the level of variation found in addition chromosome lines of wheat (Pfosser *et al.* 1995), indicating some other explanation is required.

The presence of retrotransposons has been suggested as an explanation for the three-fold change in genome size between sorghum (*Sorghum bicolor*) and maize (*Zea mays*) over the 16 million years since their divergence (Sanmiguel & Bennetzen 1998). Whether such a mechanism can be invoked to explain the variation observed in *Hieracium pilosella* populations is unknown, but the abundance of retrotransposons in the genome has been estimated to be 30-50% (Lambie 1999). The presence of "intergene retrotransposons" *sensu* Sanmiguel & Bennetzen (1998) is a possible explanation, but this may not be the most likely scenario due to the relatively short isolation times of each population.

It is likely that the genome size differentiation observed in the populations of *Hieracium pilosella* is indicative of little more than reproductive isolation between the sites, and the relatively large impact of low levels of recombination on the population structure of this species. Greilhuber (1998) states that "insufficient consideration of the boundaries of the

actually interbreeding community" often results in misinterpretation of genome size variation, and this may also be the case in finer scale nuclear DNA content measurements. Although the presence of active retrotransposons can't be eliminated, it is perhaps most parsimonious to assign the variation to a mechanism that is known to operate in this species. That the higher levels of nuclear DNA content variation are associated with the sites with obligate sexual plants present indicates that recombination is a mechanism that could be responsible for the generation of such variation.

A survey of the literature revealed numerous studies into intraspecific genome size variation, and the values for *Hieracium pilosella* for the seven field populations were compared to common commercial cultivars in studies that used comparable methods. Some studies which have dubious results have been excluded due to doubts over the methodologies used (see Greilhuber 1998 for an explanation).

Table 2.3.8. Variation in Genome size in common plant species and cultivars.

Species	**SE**	**Author, Fluorochrome, Seed Source**
Hieracium pilosella (hawkweed)	0.0209	Houliston, this work, DAPI, Wildtype Populations
Bellis perennis (daisy)	0.0036	
Raphanus sativus (radish)	0.0116	*Doležel, Sgorbatti & Lucretti 1992, DAPI, Commercial Cultivars
Lycospersicon esculentum (tomato)	0.0173	
Zea mays (maize)	0.0100	
Pisum sativum (pea)	0.0119	
Vicia faba (broad bean)	0.0156	
Allium cepa (onion)	0.0147	
Triticum aestivum (wheat)	0.0238	Pfosser, Amon, Lelley & Herberle-Bors 1995, DAPI, Addition Chromosome Lines
T. aestivum cultivars – addition lines	0.0010 - 0.0153	
Glycine max BSR 201 (soybean)	0.0053	Greilhuber & Obermayer 1997, DAPI, Commercial Cultivars
Glycine max cultivars	0.0106 - 0.0179	
Glycine max	0.0158	Rayburn, Birdar, Bullock, Nelson, Goumet & Wetzel 1997, PI, 90 Commercial Cultivars
MEAN	**0.0094**	
STDEV	**0.0052**	

Although *Hieracium pilosella* falls at the high end of the range of ploidy variation, and is certainly a lot higher than the common lawn daisy, *Bellis perennis,* used as an internal standard in this study, it does not appear to have an extremely large range of nuclear DNA content in the pentaploid state. Some species appear to have higher rates of variation in this trait than others, some such as *Allium cepa* are highly conserved worldwide (Bennett *et al.* 2000), whereas this study has found variation over very small scales in *H. pilosella.* The literature contains few references to this type of work in apomictic taxa, and the prevalence of this in asexual groups is worthy of further investigation.

II.4.1 QUANTIFICATION OF THE VIABLE POLLEN PRODUCTION OF NEW ZEALAND POPULATIONS OF *HIERACIUM PILOSELLA.*

To quantify the true potential for sexual reproduction in *H. pilosella* it is necessary to not only quantify the female function (see Chapter II.1), but also to determine the level of viable pollen production in apomictic types. A comparison between the amount of viable to non-viable pollen in apomictic and sexual biotypes is necessary to determine the investment in male function in apomictic *H. pilosella,* and to fully understand the potential for facultative sex in this species.

Numerous studies have confirmed the presence of meiotic embryo sacs in apomictic types of *Hieracium* subgen. *Pilosella* (Rosenberg 1907, Skalińska 1971, 1973, Koltunow 1993, Koltunow *et al.* 1998). Investigations into the pollen production of apomictic *H. pilosella* in Europe have agreed that these plants do produce some viable pollen (Bergström 1969, Turresson 1972, Gadella 1987, 1991b), but very few have attempted to give a quantitative measure of viability.

Although apomixis in *Hieracium pilosella* is of the autonomous type, and no pollen is required for the development of endosperm, male sterility has not been reported. The retention of pollen production is common in autonomous apomicts, with very few being reported as male sterile (Nogler 1984).

Gadella (1987) suggests that pentaploid apomictic *Hieracium pilosella* produces a high degree of functional pollen. Several pentaploid apomictic populations from across Europe were examined for their ability to act as a paternal parent in crosses with a sexual tetraploid. Gadella (1987), from the result of crosses, predicts that most of the viable pollen produced by pentaploids is diploid, as this was found to be the paternal parent in 74% of cases. Quantification of the proportion of viable to non-viable pollen was not attempted, and Gadella ruled out selfing via mentor effects (*sensu* Richards 1991) on the basis of the results of an inter-ploidy level cross.

Turresson and Turresson (1960) report experimental hybrids between apomictic pentaploid and sexual tetraploid accessions of *Hieracium pilosella* from Scandinavia, indicating again that apomicts produce viable pollen. No attempt was made to assess the proportion of viable to non-viable pollen. Turresson (1972) states the pentaploid forms "excellent pollen", but does not indicate how this was determined.

Other evidence for the production of viable pollen in *Hieracium* subgen. *Pilosella* has come from studies of self-incompatibility in the group. Krahulcová *et al.* (1999) reports that apomictic accessions of *H. aurantiacum* and *H. glomeratum* could be used as paternal parents in crosses to maternal sexual diploid *H. lactucella* and tetraploid *H. pilosella,* indicating the ability of both apomictic types to produce good pollen. Pollinating with *H. glomeratum* did result in some matroclinal progeny, indicating that the presence of foreign may have broken down the biochemical self-incompatibility of the stigma, allowing selfing to occur in the sexual accessions (i.e. mentor effects). This was not observed when using *H. aurantiacum* as a paternal parent, indicating that different levels of viable pollen production exist in apomictic members of the subgenus (Krahulcová *et al.* 1999).

Determining pollen viability

Although there are many methods available to determine the proportion of viable to non-viable pollen, this study used Alexander's stain (Alexander 1980), and the Fluorochromatic reaction (Heslop-Harrison & Heslop-Harrison 1970). These tests are not for pollen viability *per se*, but will distinguish between aborted and non-aborted pollen by differential staining of cytoplasm, or enzymatically induced fluorescence, respectively. Both of these methods require assumptions to be made between the function of the cell and viability, and whilst not absolute measures, do allow for comparison between populations. If discrepancies exist between actual pollen viability and the proportion stained, then we can assume that this will be at a similar rate over all the populations sampled. Although this may lead to an inaccurate measure of actual viability, it will allow for between population comparisons to be made.

Alexander's staining

This procedure stains for the presence of aborted and non-aborted pollen, determined by differential staining of cells. A positive test demonstrates the presence of cytoplasm, and therefore can be an overestimate of actual viability of the pollen grains (Dafni 1992). Alexander's stain can be problematic in respect to pollen wall thickness, and individual optimisation for species is often necessary. The pH of solutions in particular can alter the effectiveness of this technique (Dafni 1992).

The Fluorochromatic reaction (FCR) test for pollen viability

The fluorochromatic reaction test for pollen viability relies on the transport of fluorescein across the cell membrane, and therefore is a test for active esterases and the presence of an undamaged plasmalemma rather than viability (Dafni 1992). The mechanism for this method is the transport of the non-polar, non-fluorescent, fluorescein di-acetate across the membrane following the removal of the acetate group by esterases. The subsequent fluorescent product, fluorescein, is polar, and therefore cannot cross the plasmalemma. The presence of fluorescein in the cell is easily detected using UV microscopy, although it is possible to use a conventional light microscope with appropriate filters. Maximum light absorbance for fluorescein is between 450 and 500 nm, with emitted light at approximately 520 nm (Duncan & Widholm 1990). As dead cells have both limited esterase activity and sub-optimal plasmalemma, this technique is a useful test for pollen viability (Heslop-Harrison & Heslop-Harrison 1970). There is thought to be an excellent correlation between the presence of an intact and functioning plasmalemma and cell viability. For this reason, FDA staining is often preferred over other methods for measuring pollen viability (Heslop-Harrison & Heslop-Harrison 1970, Duncan & Widholm 1990, Dafni 1992).

II.4.2 MATERIALS AND METHODS

Samples were selected to represent the field sites used during the 1999/ 2000 season. The *Dracophyllum* flat lower and Redcliffes station sites had obligate sexual ramets only collected for the pollen study, to provide a comparison between pollen production in

sexual and apomictic plants. All other sites used plants resulting from the field crosses (Chapter II.1), but only plants produced via apomixis were used. Each sample consisted of a single capitulum from either an individual cross (apomictic plants) or an isolated ramet (sexual plants). At least 250 pollen grains were examined per capitulum, and seven capitula were examined per site.

Alexander's staining

Pollen was mounted directly in a drop of Alexander's' stain [10 ml 35% ethanol, 10 ml (1 ml of 1% solution in 95% ethanol) malachite green, 50 ml dH2O, 25 ml glycerol, 5 g phenol, 5 g chloral hydrate, 50 ml (5 ml of 1% solution in dH2O) fuchsin, 5 ml (0.5 ml of 1% solution in dH2O) orange G, 1.5 ml glacial acetic acid], covered with a cover-slip and warmed over a flame (Alexander 1980). Aborted pollen is stained green, non-aborted, red. Counts were made using an Olympus CH microscope, and the proportion of aborted to non-aborted pollen was calculated.

The fluorochromatic reaction (FCR) test for pollen viability

Pollen was removed from the anthers of fully open capitulum by washing in a 10% sucrose solution on a slide. 10 µl of 0.002% fluorescein diacetate (FDA) in acetone was added to the solution, and a cover slip added. The preparation was viewed with an Olympus BH2 microscope with an ultra violet lamp. A UG 1 excitation filter and a L420 barrier filter were used, with a U dichoric mirror. At least 250 pollen grains were counted per sample, firstly under white light and secondly under UV. The proportion of fluorescent to non-fluorescent was recorded by comparing the number of grains visible under each light source. Negative controls were prepared, without the addition of FDA, to control for the natural fluorescence of pollen lipid wall constituents. A single factor ANOVA, using site as a factor and the proportion of pollen stained as a response was carried out, to detect possible between population variation. A Least Significant Difference test was used to determine where significant differences existed between populations (Sokal & Rohlf 1981).

II.4.3 RESULTS

Alexander's staining

Alexander's stain resulted in the complete staining of almost every pollen grain examined, which indicated false positives were a problem with this method. This could potentially be due to difficulties in obtaining the correct pH or heating treatment for the pollen wall thickness. This method was abandoned, and further optimisation was not attempted, following the success of the FDA method with *Hieracium pilosella.* FDA is also possibly a better method for this type of study (Duncan & Widholm 1990, Dafni 1992).

The flurochromatic reaction

The fluorochromatic reaction detected a high degree of stainable pollen from all capitula examined. From this it can be inferred that this pollen possesses an intact and functioning plasmalemma indicating cell function. Low variation was detected between capitula, and between the sites surveyed (Table 2.4.1). There was a significant difference detected between the pollen viability of the six sites examined (P = 0.0236) (Table 2.4.2). The most notable difference was the significantly lower level of pollen viability at the Chilton valley site. This site also had the highest standard error, with two of the seven capitula examined having much lower viability counts than the others (samples 2116, 2126).

Negative controls resulted in very low levels of pollen fluorescence, which were easily distinguished from the amount of fluorescence following the addition of FDA.

Table 2.4.1 Percentage Pollen Viability as determined by the FDA assay, field sites 1999 / 2000 (all values ± std error).

Site	% Stained pollen
Dracophyllum flat lower	83.57 ± 0.055
Dracophyllum flat upper	70.23 ± 0.128
Cass flats	72.84 ± 0.268
Chilton valley	56.32 ± 0.421
Little river	77.29 ± 0.084
Redcliffes station	77.28 ± 0.152

Table 2.4.2 Single Factor ANOVA, Analysis of Variance Table, *Hieracium pilosella* Pollen Viability as a proportion.

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Site	5	0.3039856	0.06079713	2.981956	**0.0236**
Residuals	36	0.7339803	0.02038834		

Table 2.4.3 Least Significant Difference test, Pollen Viability by Site. Those that share a line beneath them are not significantly different at the level indicated (solid line $\alpha = 0.05$).

Draco flat lower	Little river	Redcliffes	Cass flats	*Draco* flat upper	Chilton valley
_____		_____			
	_____		_____	_____	

There was no significant difference found between the pollen viability of the sexual plants from the *Dracophyllum* flat lower and Redcliffes sites, and apomictic plants at the Little river site. The Cass flats and *Dracophyllum* flat upper sites did not have significantly different pollen viability to the Little river and Redcliffes site. Chilton valley was the only site found to have a significantly different level of pollen viability to both of the sexual accessions (*Dracophyllum* flat lower & Redcliffes).

II.4.4 DISCUSSION

Pollen viability of both sexual and apomictic accessions of *Hieracium pilosella* appeared to be high. As detected by the fluorochromatic reaction, presence of an intact plasmalemma and functioning esterases were found in the majority of pollen grains examined, indicating a substantial investment in the paternal function of this species. Of particular relevance is the finding that levels of pollen viability in three of the four sites comprised of facultative apomictic individuals is not significantly different to those found in the obligate sexual *Dracophyllum* flat lower and Redcliffes station accessions. The lower level of pollen viability found at the Chilton valley site is due to two of the

capitula having considerably lower pollen viability, approximately 20-25%. Larger sample sizes would be necessary to determine the extent of the lower viability at this site.

Male investment is common in other apomicts, including autonomous taxa (Mogie 1992), and this facilitates the facultative nature of apomixis in these species. It is also clear that pollen availability in predominantly apomictic populations of *Hieracium pilosella* should not be limiting, assuming pollination vectors are present (see Chapter II.1). The ability of *H. aurantiacum* to pollinate both sexual and facultative apomictic *H. pilosella* (see Chapter II.1) illustrates the ability of plants of this subgenus to produce viable pollen, even when aneuploid in the case of A3.4.

This finding further indicates that the potential for sexual reproduction does exist in predominantly apomictic populations of *Hieracium pilosella.* Investment in male function does not seem to decrease significantly with a shift to asexuality, indicating perhaps an evolutionary advantage in this strategy. The retention of pollen production may indicate that there is some fitness potential to be had by producing male gametes, either to fertilise facultative apomicts, or obligate sexuals. The contrasting view of this is that there is either insufficient variation in this trait, or insufficient cost, for this to be selected against.

Chapter III. RELATIONSHIP BETWEEN THE FREQUENCY OF SEXUAL REPRODUCTION AND POPULATION GENETIC VARIATION

III.1.1 INTRODUCTION

Although there is potential for sexual reproduction in New Zealand populations of *Hieracium pilosella* (Chapman & Bicknell 2000, Houliston & Chapman 2001), it is unclear whether this contributes to population genotypic variation. This chapter aims to determine whether sex does play a role in structuring field populations of apomictic *H. pilosella* by comparing population genotypic variation and the frequency of sex, as measured in chapter II.1, at selected sites. Also to be tested is whether it is possible to determine the contribution of sex to population structure by using simple molecular methods and compatibility analysis (Mes 1998).

Population variability in apomictic species

Clonal plant species, particularly those that are also apomictic, have often been assumed to possess a limited amount of, and limited potential to generate, variation. For this reason they have often been labelled as evolutionary "dead-ends" (Stebbins 1950, Asker & Jerling 1992, Mogie 1992). Studies since the advent of molecular techniques have shown however, that the presence of high levels of genotypic variation, comparable to sexual populations, is not an uncommon phenomenon in predominantly asexual populations. A review of 27 studies of 21 clonal plant species by Ellstrand and Roose (1987) concluded that "intermediate diversity" and an "even distribution" of diversity among populations were the most common patterns. All but two of the 27 studies detected multiple genotypes, and most genotypes (75.8%) were restricted to a single population. Also noteworthy was their finding that large clones, present in more than 75% of the populations, were uncommon, comprising less than 2% of genotypes in over half of the species. Additionally, there was a relationship ($R^2 = 0.34$, $p = 0.05$) between the number of characters scored and the number of genotypes detected. This indicates

that many of these studies perhaps underestimated the number of genotypes present due to a lack of characterisation of the individual samples (Ellstrand & Roose 1987).

A later review looked at 40 studies of genotypic diversity using 45 species (Widén *et al.* 1994). Only two of the studies examined used DNA techniques (Restriction Fragment Length Polymorphisms and Random Amplified Polymorphic DNAs), while all of the others used isozymes. In contrast to Ellstrand and Roose (1987), uniclonal populations were relatively common, found in 22 of the studied species. This may indicate a limitation of the isozyme method for this type of work. Another concern expressed by Widén *et al.* (1994) was that comparison between studies using these types of methods is often problematic due to the different sampling methods employed. Widén *et al.* (1994) tried to control for this by including studies that also describe the spatial arrangement of populations. Only five studies were found where all the ramets present in a plot or subset of the population were examined, and a further nine studies were sampled on transects or grids. There were eight studies where a single ramet was collected from a patch as a representative sample, and the remaining 26 studies were comprised of random sampling. All of these factors make drawing strong conclusions from review papers like this very difficult. What is clear is that plant species with predominantly clonal reproductive systems can possess complex population structures.

It should be noted that both Ellstrand and Roose (1987) and Widén *et al.* (1994) included species that were clonal due to vegetative spread or the production of bulbils, not just those that are truly apomictic *sensu lato*. Of the species included by Ellstrand and Roose (1987), nine were apomictic, of which three also spread vegetatively. Widén *et al.* (1994) also included nine species that were apomictic, none of which spread vegetatively. In both studies it appeared that there was no difference between the diversity of the populations of these species and the other reproductive types included in the study. The failure to distinguish between true apomicts and species with vegetative spread or permanent translocation heterozygotes is usual in such studies (see also McLellan *et al.* 1997). The inclusion of species that produce outcrossed seed but were clonal due to growth strategy further confuses the interpretations of these reviews. How such studies can be interpreted in regard to the structure and potential for evolution in apomictic species is difficult to determine.

Both Ellstrand and Roose (1987) and Widén *et al.* (1994) identify two species that are interesting due to the absence of multiple clones; *Taraxacum obliqum* an aposporous apomict, and *Gaura triangulata,* a permanent translocation heterozygote. Another exception to this pattern was discovered in *Taraxacum albidum*, a pentaploid, obligate agamospermous apomict, indigenous to Japan (Menken & Morita 1989). 109 plants from 12 populations representing the entire range of this species were collected, and isozyme profiles compared for 10 different systems, resulting in 19 loci. In all but one individual, the plants were identical in banding pattern, and the plant that did differ was only variable at one locus. It was also interesting to note that heterozygosity was high, with 13 out of 19 loci being fixed heterozygotes. The authors of this study admit that while their sample sizes were small, and the number of genotypic characters low, they did include the entire geographic range of the species.

More recent investigations into levels of genetic variation have found similar patterns to those described by Ellstrand and Roose (1987) and Menken *et al.* (1995). Menken *et al.* (1995) looking at variation in populations of *Taraxacum* section *Ruderalia,* a mixed amphi-apomictic species complex, found that apomictic populations possessed high diversity, and shared all major isozyme polymorphisms with the sexual populations. It is also interesting to note that both sexual and apomictic populations had allele frequencies generally agreeable with Hardy-Weinberg equilibrium. Lyman and Ellstrand (1998) compared levels of isozyme diversity in populations of the obligate sexual *Taraxacum californicum* with obligate apomictic *T. officinale* growing in North America. *T. officinale* possessed approximately half the genotypic diversity of *T. californicum*, and population differentiation in the two groups was comparable. However, it is important to note that this study had an order of magnitude difference in sampling regimes of the two reproductive types, the apomict being much more widespread. Durand *et al.* (2000) found that clones of the facultative apomict *Hyparrhenia diplandra* in West Africa were typically no greater than 15m wide, and suggested the low rate of sexual reproduction (0.5 %) in this species was a possible explanation for this diversity. A similar finding was reported by van der Hulst *et al.* (2000), who used AFLPs (Amplified Fragment Length Polymorphisms) to detect genotypic diversity in populations of triploid *T. officinale* isolated from diploid sexuals. Limited clonality was observed, with some

individuals having identical AFLP fingerprints, although most genotypes were detected only once.

Studies of this nature in *Hieracium* are not so numerous, however Shi *et al.* (1996) reported low variation in four species of *Hieracium* section *Alpina*. *H. holosericeum* was found to comprise only a single genetic clone in Britain as detected by isozyme and RAPD markers. Two of the remaining species in this study (*H. tenuifrons* and *H. calenduliflorum*) possessed very low levels of variation, which was concordant with geography for RAPD markers, and population with isozymes. RAPD markers showed differences in *H. alpinum* populations between Scotland and Switzerland but no within-country variation, whereas isozymes found considerable within-population differences. The differences in isozyme profiles in the variable species in this study were attributed to the presence of several different, morphologically detectable, races (Shi *et al.* 1996).

Population structure of *Hieracium pilosella* in New Zealand has recently been examined by Chapman *et al.* (2000) (as *Pilosella officinarum*). Inter-Simple Sequence Repeats (ISSRs) were employed to examine the amount of clonality present in five field populations of *H. pilosella* from the South Island of New Zealand. 150 ramets were sampled in total, and 39 different ISSR phenotypes identified. All sites were found to contain multiple genotypes, although two clones were found to be present at all of the sites. These two clones represented almost one half (48%) of all of the populations sampled. All sites also were found to have unique genotypes present that were not found at any of the other sites. The divergence amongst clones, however, appeared to be low with most unique individuals only differing by one band. Although Chapman *et al.* (2000) employed six different ISSR primers, in total only 32 scorable bands were present. ISSR phenotypes typically have considerably more scorable bands than this (G. Houliston *pers obs.*), which indicates that Chapman *et al.* (2000) may be a conservative measure of the possible population variation present in this species. As ISSR has already been shown to be an adequate method for the detection of population variation in this species (Chapman *et al.* 2000), it will be employed in this study.

The advantages of the ISSR technique have already been described in chapter II.2.1, and will not be discussed further here.

Assigning a mechanism to the generation of population variability

All of these population studies demonstrate that genotypic variation is typically higher than expected, assuming an entirely apomictic breeding system. Because of this disparity between assumed reproductive mode and population variation, potential mechanisms for the generation of this variation are often suggested (see Vrijenhoek (1990) for a review). The two most commonly cited potential explanations are residual levels of sexual reproduction, and mutation. Although mutation is widely provided as a possible explanation (see Ellstrand & Roose 1987, Menken & Morita 1989, Asker & Jerling 1992, Menken *et al.* 1995), little more than anecdotal evidence exists; Richards (1996a) perhaps the exception. It should also be noted that the widely cited examples of Sørensen and Gudjonsson (1946) [in Richards 1996a] have been largely put down to karyological errors (Richards 1996a). Although the role of mutation as a path for the generation of variation in apomicts should not be discounted, there is growing evidence that low levels of sexual reproduction are a more likely explanation (see Richards 1996b).

The lack of empirical testing of these ideas for the generation of variation in clonal plant species and the lack of knowledge of the potential contribution of these mechanisms has drawn criticism (Eckert 1999). Recent works have begun to address this problem however, at least in the importance of the role of sexual reproduction in these species.

King (1993) in a study of *Taraxacum* naturalised in North America, suggested that the population structure detected using both restriction enzyme analysis of ribosomal DNA (rDNA) and chloroplast DNA (cpDNA) was more consistent with hybridisation between existing genotypes than mutation. Studies of this nature are useful to measure population variation, and although patterns of variation can be indicative of a particular mechanism, it is not possible to exclude either explanation.

Schneller *et al.* (1998) suggests that mutation is a more likely mechanism for the pattern of variation observed in the apomictic fern *Dryopteris remota*. Although it is probable that the low level of variation observed amongst individuals is best explained by mutation, it is unsatisfying to be unable to assign a probability to such a conclusion.

Mathematical models have been constructed to attempt to quantify the relative contributions of sex to the variation in clonal plant species. Invariably, these models have assigned all genetic variation to sex, and also require complete knowledge of the loci under investigation (Marshall & Brown 1974, Harada *et al.* 1997). The application of these models is almost always further restricted by the assumptions made in the construction of the model. Other works have attempted to determine how effective a marker system is at detecting genotypes, to allow a probability to be assigned to how likely it is that two identical genotypes are in fact the result of clonal processes. By calculating the chance of the same marker pattern arising randomly via sex it is possible to calculate how likely it is that a pair of individuals with the same marker phenotype are clonal (van der Hulst *et al.* 2000). While this does not allow a measure of how much sex has contributed to the population, it does give an indication of the population structure and whether individuals can be declared unique on marker differences.

Compatibility analysis

Recently, cladistic analysis methods have been developed to determine the likelihood of fingerprinting patterns from PCR techniques with dominant markers being explained by either recombination or mutation (Mes 1998, van der Hulst *et al.* 2000, Mes *et al.* 2002). These techniques use "component compatibility analysis" to determine if the differences between genotypes can be explained by the addition of mutations. This is illustrated by a comparison of potential patterns observed in pair-wise, binary characters. If mutation is the sole mechanism, only three of the four possible combinations of characters are possible in a lineage, excluding the presence of back mutation (Le Quesne 1969). If all four character combinations are present, this is deemed an incompatibility, and is most parsimoniously explained by recombination. Mes (1998) states that this is most likely an under-estimate of incompatibility as only pair-wise comparisons are made, if multiple characters are included simultaneously then estimates will be higher. The total number of such incompatibilities can be summed for the entire matrix, and the amount of recombination gauged. While this method is a useful tool, it is very much an approximation of the importance of each mechanism. Mes (1998) even goes so far as to describe this as a "rather crude" way to determine the predominant mode of reproduction, however in systems where sexual events are rare it can be a useful method (cf. Brookfield 1992). Further advantages of incompatibility analysis is that it is not necessary to have

an "allelic interpretation", making it possible to apply to both polyploids and dominant data (van der Hulst *et al.* 2000). Incompatibility analysis will be applied to the data sets of this study to try to determine the relative importance of recombination and mutation in generating the patterns of population variation observed.

To determine if there is any correlation between residual sexual reproduction and population variation, three populations with differing levels of sexual reproduction will be compared using ISSR profiles, to determine if a relationship exists between the frequency of sex and genotypic diversity. The results of this will be compared to the predicted values for the contribution of sex to each of the populations from the compatibility analysis (Mes 1998), to determine whether this is a robust method for inferring such mechanisms.

III.1.2 MATERIALS AND METHODS

Selection of sites and sampling

The Chilton valley, Little river and Cave stream field sites were selected to provide a contrast in residual levels of sexual reproduction. The frequencies of sexual reproduction, as determined by the methods in chapter II.1, for the three years recorded can be seen in Table 3.1.1.

Table 3.1.1 Frequencies of Sexual Reproduction at the Chilton valley, Little river, and Cave stream field sites (no. sexual / total progeny).

	1998 / 1999	1999 / 2000	2000 / 2001	Total
Chilton valley	(5 / 794)	(7 / 535)	(12 / 984)	(24 / 2313)
	0.63 %	1.31 %	1.22 %	1.04 %
Little river	(45 / 1676)	(17 / 629)	(10 / 544)	(72 / 2849)
	2.68 %	2.70 %	1.84 %	2.53 %
Cave stream	(1 / 586)	Not	(4 / 270)	(5 / 856)
	0.17 %	Sampled	1.48%	0.58%

28 individual rosettes were sampled at the three field sites and DNA extracted following the method described in chapter II.2. All rosettes were situated at least 500 mm from each other and within the boundaries of the existing field sites.

ISSR methodology

Inter-Simple Sequence Repeats were performed using a modification of the protocol in chapter II.2.

ISSR PCR was carried out as a 25 μl reaction per sample:

Volume (μl)	Constituents
2.5	10 x Taq polymerase PCR buffer (Boehringer Mannheim)
1.25	10 mM dNTPS (Boehringer Mannheim)
3.0	25 μM Magnesium Chloride (Boehringer Mannheim)
1.0	10 mM Primer
0.2	Taq DNA polymerase (Boehringer Mannheim)
16.05	dH20
1.0	suspended DNA product
25.0	

Primers used were from the University of British Columbia Biotechnology Laboratory Microsatellite primer set 9 (see Table 3.1.2). Primers were those used by Chapman *et al.* (2000), with the exception of 897 which did not detect any polymorphism in the five populations sampled in this study. These primers were selected as the most informative of the 100 screened by Chapman *et al.* (2000) for their population study of *Hieracium pilosella* (as *Pilosella officinarum*).

The reaction was amplified in a PTC-200 Thermal Cycler (MJ Research). Initial denaturation was 4 minutes at 93°C, followed by cycles of 93°C for 20 seconds, 48°C for 60 seconds, 72°C for 20 seconds, for a total of 41 repeats. Final extension was 72°C for 4 minutes and the reaction was then halted by a constant 4°C treatment.

Electrophoresis and scoring was carried out as per chapter II.2, and the analysis performed using MVSP version 3.1a (Kovach Computing Services 1999). Additional diversity indices were calculated using the descriptive statistics function of TFPGA version 1.3 (Miller 1997). Jaccard's similarity (see Chapter II.2) was used in all cases as this method only takes into account positive matches between bands, and as missing bands in ISSR profiles can be for several different reasons (Weising *et al.* 1995), this is an appropriate method. UPGMA (unweighted pair-group method using arithmetic average) dendrograms were constructed using a Jaccard's similarity matrix (see Figures 3.1.2 - 3.1.4). UPGMA was selected as it gives equal weight to each point in each cluster. This assumes that the clusters in the data are of approximately equal size, and no hierarchical sampling has occurred, with no one cluster being sampled more or less than others. It treats the cases as independent, random samples, as is appropriate for the sampling method used (Kovach Computing Services 1999).

The average percentage of seed produced sexually was regressed against the marker heterozygosity as a measure of population variation. The regression slope and significance level was compared to determine if a relationship exists between the levels of residual sexual reproduction and population variation.

Compatibility analysis

Matrix incompatibility for each of the sites was calculated using PICA version 4.0 (Wilkinson 2001). The JACTAX.EXE function was used to calculate the incompatibilities present in the data set due to all genotypes, and to sum the total for the data set. The genotypes responsible for the greatest number of incompatibilities were successively deleted from the data set, following the method of Mes *et al.* (2002). The analysis was then repeated with the next most conflicting genotype removed until all compatibility was resolved. This allowed both the number of genotypes in the population that could be explained by compatibility, and the number having to be removed to achieve this, to be calculated. The probability that a particular ISSR marker had no less incompatibility with other characters in the data set than a random permutation of character frequencies (Le Quesne probability, [Le Quesne 1969]) was examined using the LQPROB.EXE of PICA version 4.0 (Wilkinson 2001). This will determine whether

marker distributions are significantly more conservative than random permutations, and therefore are more indicative of clonal reproductive modes (Mes *et al.* 2002).

III.1.3 RESULTS

ISSR population genotypic variation

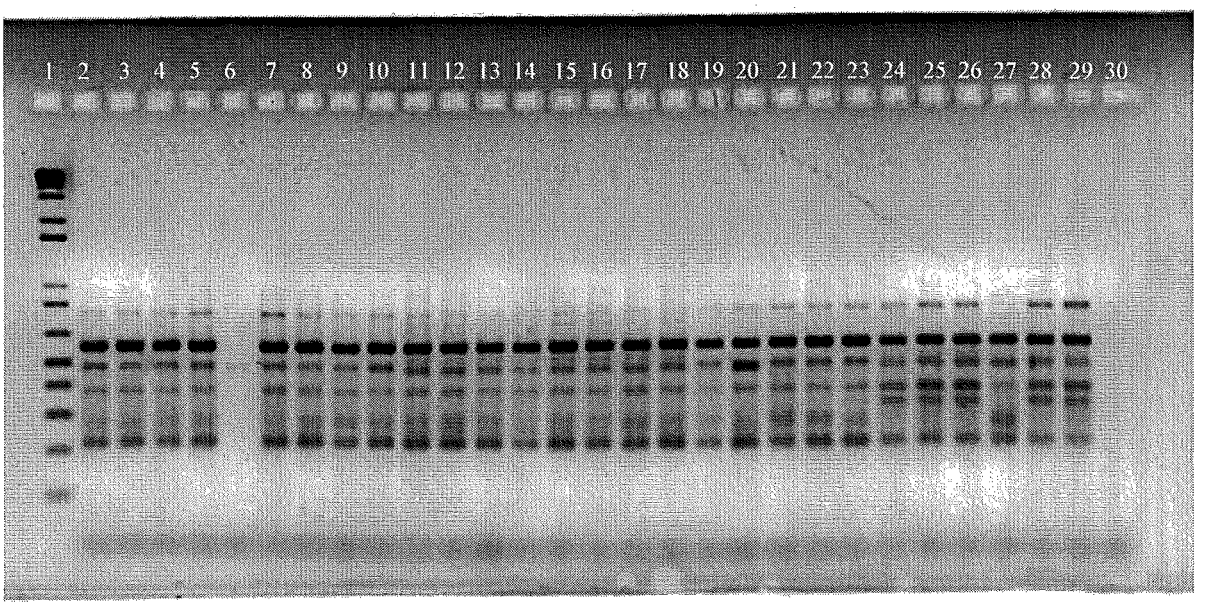
27 samples from the Chilton valley and Cave stream, and 28 from Little river were amplified by the five primers used in this study. ISSR primers produced a high degree of scorable bands, although the amount of polymorphic bands per primer was variable (Table 3.1.2). Only one primer (866) was found to produce no polymorphic bands over the three populations examined.

Table 3.1.2 Primer Sequences and Summary of ISSR bands scored

Primer No.	Primer Sequence (5′- 3′)	No. of bands per primer	No. of polymorphic bands
UBC 900	ACT TCC CCA CAG GTT AAC ACA	8	6
UBC 895	AGA GTT GGT AGC TCT TGA TC	10	10
UBC 866	CTC CTC CTC CTC CTC CTC	4	0
UBC 845	CTC TCT CTC TCT CTC TRG	13	12
UBC 822	TCT CTC TCT CTC TCT CA	9	9

An example of an ISSR gel can be seen in Figure 3.1.1. Colour has been inverted to improve resolution of the bands. UPGMA dendrograms for the three populations can be seen in Figures 3.1.2 to 3.1.4. Individuals were coded by a letter and then the number of the collected sample, (Little river: LR 1 - 28, Chilton valley CV 1 - 27, Cave stream CS 2 - 30 (excluding 5 & 8). Genetically identical (clonal) individuals were found at all sites, although there were no shared genotypes between sites. If all samples were included in a dendrogram, individuals mostly clustered by population, with the exception of a few unique (one individual of the genotype recorded) individuals (See Appendix 6.1).

Figure 3.1.1 ISSR Reaction, Little river population, UBC Primer set 9, 900.



Key; LANES: 1- 1 kb ladder, 2 to 29, Little river samples *H. pilosella* 1 to 28; lane 6- number L5 is a failed reaction, 30 – negative control.

Figure 3.1.2 UPGMA dendrogram for the Little river site, ISSR data, Jaccard's coefficient

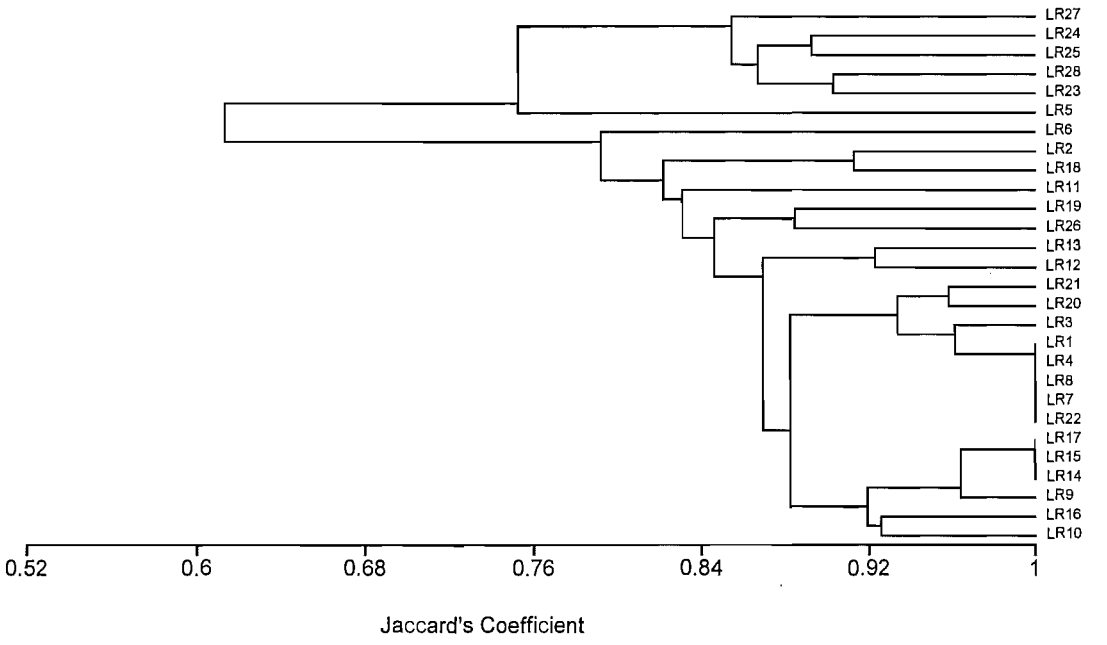


Figure 3.1.3 UPGMA dendrogram for the Chilton valley site, ISSR data, Jaccard's coefficient

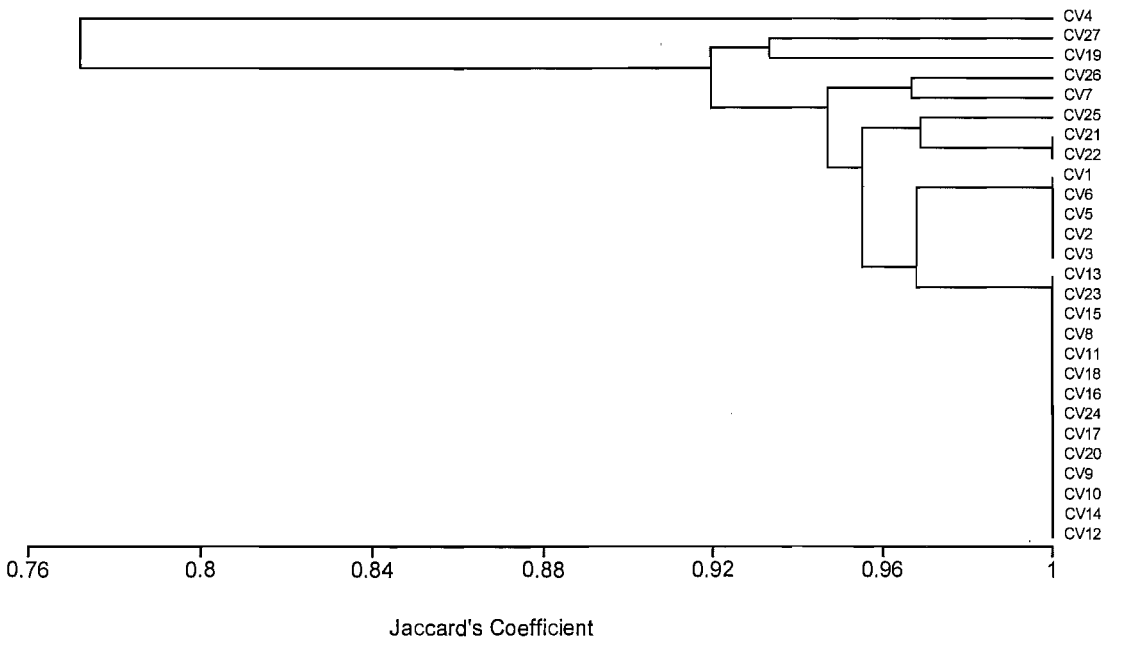
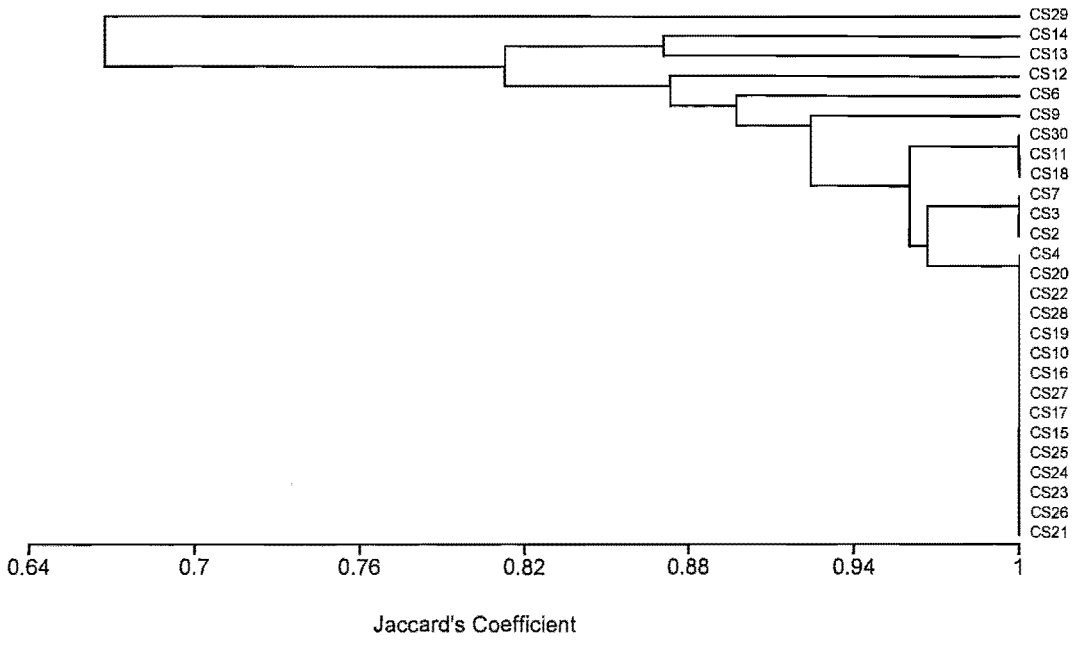


Figure 3.1.4 UPGMA dendrogram for the Cave stream site, ISSR data, Jaccard's coefficient



Measures of relative population genotypic diversity can be seen in Table 3.1.3. The Little river site, as sampled, was comprised of 22 genotypes, whereas the Chilton valley and Cave stream sites had only nine. This difference in variation is also reflected in the percentage of polymorphic bands and the mean marker heterozygosity of the populations (Table 3.1.3).

Table 3.1.3. Summary of ISSR band characteristics for each site

	Little river	Chilton valley	Cave stream	Total
No. of samples	28	27	27	82
Total no. of bands	38	36	35	44
No. variable	28	12	15	36
No. unique to site	5	3	1	9
% polymorphic bands	73.7	33.3	42.9	81.82
% of all bands present	86.4	81.8	79.6	100.00
No. of genotypes	22	9	9	40
No. of clones	2	3	3	8
Mean heterozygosity*	0.1930	0.0590	0.0926	NA

*As ISSR are a dominant marker, heterozygosity is calculated on marker frequencies, as is the convention in such analysis, and does not represent true loci heterozygosity. The presence of a band is assumed to represent the dominant genotype at a locus while the absence of the band is scored as the homozygous recessive. The frequency of the recessive allele is estimated as the square root of the frequency of missing bands a locus, and therefore assumes Hardy-Weinberg equilibrium applies (Miller 1997).

Comparison of population variation and the frequency of sex

Regression between the level of the average percentage of seed produced sexually under field conditions over the three years (two only for Cave stream), and marker heterozygosity at the three sites produced a significant, positive, relationship (Table 3.1.4). Seasonal average percentages of sexually produced seed per cross were used in the regression as a measure of the proportion of seed produced at a site for potential recruitment, that was different from existing genotypes. The relatively low range in the proportions of seed produced sexually by site meant that data transformation was not warranted (Zar 1996). Arcsine transformation did not increase the normality of the data set and very slightly decreased the fit of the regression, indicating that data transformation was not desirable.

Table 3.1.4 Regression of Percentage of Sexually produced Seed by Season and Marker Heterozygosity

Coefficients:

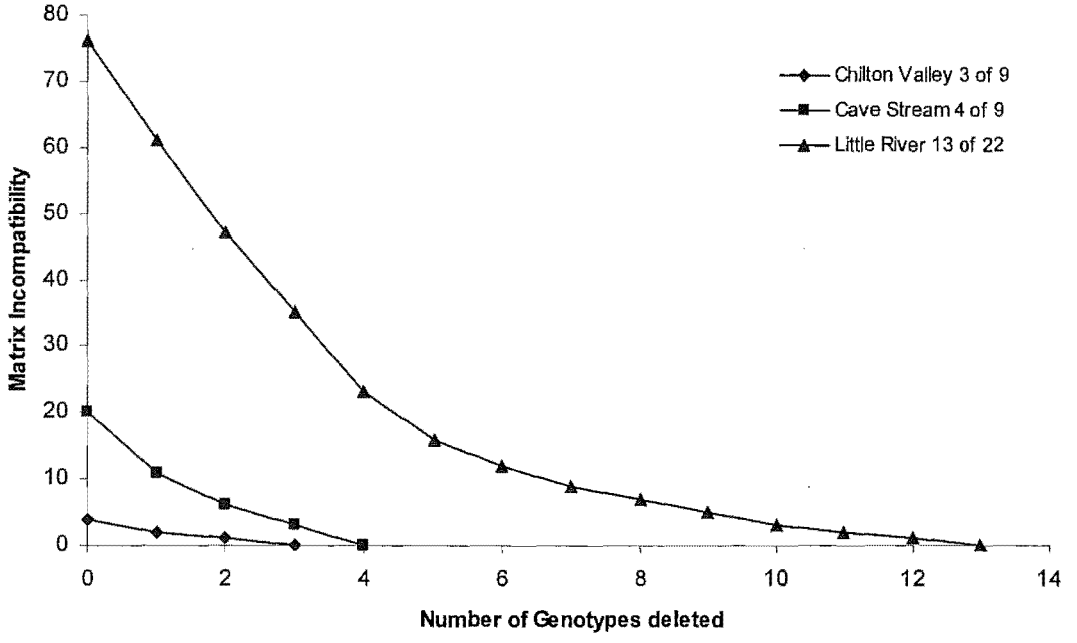
	Value	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.2476	0.6182	-0.4005	0.703
heterozygosity	13.5600	4.7539	2.8524	**0.029**

Residual standard error: 0.7839 on 6 degrees of freedom
Multiple R-Squared: **0.5756**
F-statistic: 8.136 on 1 and 6 degrees of freedom, the p-value is **0.029**

Compatibility analysis

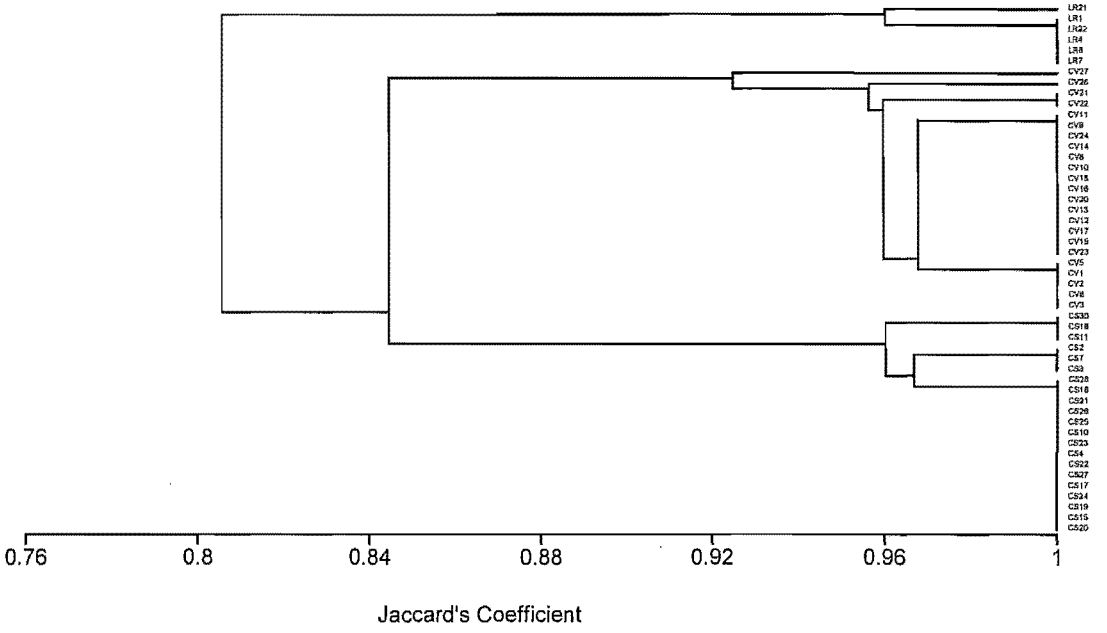
The Little river site displayed considerable incompatibility, with 59% of the genotypes having to be removed to allow complete compatibility of the data set (see Figure 3.1.5). The remaining two sites had much lower levels of initial incompatibility, and required the removal of substantially less genotypes to gain complete compatibility (see Figure 3.1.5). The continuous decline of matrix incompatibility as genotypes were successively removed indicates that no groups of genotypes are more or less incompatible with each other than any other groups or individuals (van der Hulst *et al.* 2000).

Figure 3.1.5 Graph of character incompatibility.
Reduction of Matrix incompatibility upon successive removal of genotypes from each of the three data sets.



If data for all three sites was combined, considerably more incompatibility was found in the data set. A total of 30 genotypes had to be removed from the matrix to gain compatibility, leaving two from Little river, three from Cave stream and five from Chilton valley (see Figure 3.1.6). A UPGMA dendrogram of these genotypes shows the "chaining" pattern as assumed in the analysis, and also that the genotypes retained geographic concordance (Figure 3.1.6).

Figure 3.1.6 UPGMA dendrogram of the compatible genotypes, all sites combined.



The ability to find compatibility when all three data sets are combined indicates a common origin to the populations, with subsequent differentiation via sexual reproduction and mutation. That higher compatibility is found when the data sets are analysed individually compared to the pooled data set indicates that sexual events have been followed by mutation at each of the populations.

Le Quense probability (Le Quesne 1969, Wilkinson 2001) of the marker distributions was also compatible with sexual reproduction being responsible for the population structures observed. All informative characters for incompatibility at both the Cave stream and Chilton valley sites had Le Quense probabilities substantially higher than p =

0.05, indicating that distributions of these markers were not significantly different to the results of the 1000 random permutations. Of the informative characters from the Little river site, only five of the 20 had Le Quense probabilities of $p < 0.05$ (bands 9, 12, 26, 28, 32). Band 9 was informative for the Chilton valley site with a Le Quense probability of 0.947, and band 26 was informative for the Cave stream site ($p = 0.875$). Bands 12, 28 and 32 were not informative for either of the two remaining sites.

III.1.4 DISCUSSION

Population variation and the frequency of sex

Hieracium pilosella was shown to have a complex population structure as detected by ISSRs (see also Chapman *et al.* 2000 as *Pilosella officinarum*). Unlike many other studies of this nature (see Ellstrand & Roose 1987, Widén *et al.* 1994 for reviews), this work includes a measure of the potential for sexual reproduction in the populations examined. This allows the use of a statistical test of a relationship between sex and population structure, and the ability to assign variance in the population structure to this trait. In this example, an R^2 of 0.5756 indicates that approximately 58 % of the total variation in population variation was predicted by the level of residual sexual reproduction ($p = 0.029$). This indicates that population variation depends on the level of residual sexual reproduction, but due to the use of regression it is incorrect to state a deterministic or causal relationship between the two (Howell 1992, Zar 1996).

The presence of unique (often termed "private") markers (see Table 3.1.3) in the populations indicates that the populations are genetically isolated from each other. Unique alleles may indicate sorting of genotypes following establishment of original populations at each of the sites, different origins for each of the populations, or differentiation following founder events. Due to the levels of variation, and the findings of the compatibility analysis (see below), it is likely that recombination has played an important role in the generation of novel genotypes.

All three populations contained genotypes with multiple individuals (clones). As samples were collected at least 500 mm apart this means that either relatively large vegetative clones were present or seed produced via apomixis from existing on-site clones has been recruited. Large clones dominated both the Cave stream and Chilton valley sites, although both sites also possessed unique individuals. The absence of genotypes found in more than one population further demonstrates the level of population differentiation. This can also be seen when all sites are combined in a single dendrogram, with strong divisions between populations (see Appendix 6.1). The absence of "conservative informative characters" (van der Hulst *et al.* 2000) in two of the populations means that Le Quense probabilities for interpreting the divergence of populations by looking at the structure of these most conservative but informative markers in the populations is problematic. However, as two of these characters from the Little river site are informative, but not conservative in other populations further indicates that these populations have undergone divergence. Due to the difference in distribution of these characters in the three populations, recombination is the best explanation for such a pattern (van der Hulst *et al.* 2000).

Compatibility analysis

The use of compatibility analysis can tentatively differentiate between the effects of sexual reproduction and other mechanisms such as mutation (Mes 1998). The results of this analysis for each of the three populations indicates that sexual reproduction has contributed to the generation of variation to different extents at each of the sites. The low number of genotypes that had to be removed from the Chilton valley to achieve compatibility indicates that only three of the genotypes detected can be considered as being of sexual origin. Conversely, the high proportion of genotypes having to be removed from the Little river site shows that sexual reproduction has played a very substantial role in the structuring of this population.

There was almost complete concordance between the estimate of the amount of recombination as calculated using compatibility analysis and the potential for sex as measured by the artificial pollination experiment. Due to the low number of incompatibilities recorded, it was difficult to discern the differences in the frequency of hybridisation as measured in the field between the Chilton valley and Cave stream sites

using compatibility analysis. That the incompatibility was higher at Cave stream than Chilton valley, but the potential for sex was lower, indicates that sampling regimes are possibly important for the use of this method. This technique may be more applicable to larger studies, with both larger sample sizes and geographic ranges. However, even in the small populations in this study, both numerically and spatially, compatibility analysis provided a good estimate of the contribution of sex to the populations. It is also possible that the potential for sex as measured by the field crossing experiment is not fulfilled at the sites to the same degree due to differences in seed recruitment. Regardless, it appears that in this system compatibility analysis agrees to a greater extent with the measurement of the frequency of sexual reproduction used in this study, indicating that it is a robust, albeit heuristic, tool for population analysis.

The amount of compatibility in all three of the populations indicates that mutation has played a role in the structuring of these populations. Unfortunately, in assigning mechanisms to the generation of population variation, only "heuristic" methods such as this are available (Mes 1998). In some studies the role of mutation is more definite (see Schneller *et al.* 1998), although the low level of incompatibility at the Chilton valley and Cave stream sites is indicative of this. The level of incompatibility is less than Mes *et al.* (2002) and van der Hulst (2000) detected in their studies of *Taraxacum*, indicating that sex is less frequent in *Hieracium pilosella,* although this study is over a much smaller geographic scale than either of these other works. Both *Taraxacum* studies also employed AFLPs, generating many more markers that ISSR and therefore more opportunity for character incompatibility. Van der Hulst (2000) also included over twice as many samples than the present study. Further investigation will be required that employ this method with a range of markers to better understand the level of resolution that this technique provides. Studies that also include a measure of sexual reproduction in facultative taxa, as this work does, will also improve the understanding of the resolution that this method provides. Whilst a measure of sexual reproduction as included in this study can not be directly extrapolated to population variation due to possible recruitment mechanisms, it does at least give a better understanding of the potential for the generation of population variation.

The findings of the compatibility analysis for the combined data set are interesting in that it was possible to find compatibility whilst retaining genotypes from all sites. This may indicate a common genetic origin, perhaps the same genetic founders established at each site and have subsequently undergone differentiation. Recombination, mutation and genetic drift, due to the relatively limited effective population sizes, have most likely played a role in this. That the Little river site was more distinct in the UPGMA dendrogram, and is also the most distant geographically, is consistent with a longer temporal isolation from the other populations. The identification of patterns indicative of both recombination and mutation within the combined data set is further evidence of the differentiation at the population level that has been observed in *Hieracium pilosella* (see also Chapman *et al.* 2000 as *Pilosella officinarum*, Chapman & Brown 2001).

Variation in population genotypic diversity

The levels of marker variation at the Little river site are higher than the other two sites examined (0.1930, Chilton valley 0.0590, Cave stream 0.0926). This would indicate that another explanation, other than differences in level of residual sexual reproduction, might be necessary to account for the population structure of this site. The frequency of sexual reproduction at the sites is not proportional to the marker variation observed and therefore although the fit of the linear regression equation is significant, the variation at the Little river site is too high if all other mechanisms at the three sites are equal.

Other potential explanations for the generation of the relatively complex population structure at the Little river site, compared to Chilton valley and Cave stream, are possible. The most parsimonious, given the level of potential for residual sexual reproduction at this site, is an elevated level of seed recruitment in comparison to the other sites, either total seed recruitment, or selection for outcrossed individuals. As there has been considerable variation observed in the amount of seed recruitment in field populations of *Hieracium pilosella* [standard errors of seedling density per $100m^2$ ranging from 2.48 to 4.64; Rose & Frampton (1999)], this could be a possible explanation if seed recruitment was considerably higher at this site. Selection for outcrossed progeny is also possible, but more difficult to determine. The Little river site has very different vegetation and soil type to the other two sites, so ecological differences may be responsible if this has occurred.

The origin and age of the *Hieracium pilosella* populations at the Little river site are unknown, but it is likely that they are of different, possibly more recent, origin than the Waimakariri sites due to the geographic isolation. The time of colonisation for all three sites will be very recent, most likely within the last 110 - 120 years, making it unlikely that temporal scale is important. It is possible that the Little river site had a more diverse founder population than the other sites, or at least more of the founding genotypes survived (less genotype sorting). Lack of information on the origin of the Little river population precludes any conclusions to be drawn on this.

Different mutation rates in *Hieracium pilosella* at the three sites examined are a theoretical source of further genotypic variation. Although this has been widely cited as a potential explanation for variation in apomictic populations (see Ellstrand & Roose 1987, Menken & Morita 1989, Asker & Jerling 1992, Menken *et al.* 1995), few empirical studies exist. There is the possibility of varying rates of mutation in *H. pilosella* among the three populations, however it is unlikely that there would be strong enough differences in rate to explain the differences in population structure. The much higher diversity of genotypes at the Little river site is responsible for the incompatibility observed, and other work has also stated that such a pattern is not indicative of mutational events (King 1993).

The presence of tetraploid individuals at the Little river site (see Chapter II.3) may also contribute to the variation present in this study, although these were also found at the Chilton valley site in 1999 / 00. Although it has been shown in chapter II.3 that apomictic tetraploids do not possess higher levels of residual sexual reproduction than pentaploids, the presence of tetraploids at the site may be indicative of past sexual events, and the production of this ploidy level through facultative sex. The higher incompatibility at this site would also support this conclusion.

Evolutionary potential of *Hieracium pilosella*

The relatively high variation detected in this study for all three populations examined is not unusual in apomictic taxa (see Chapter III.1.1). Despite recent investigations into this phenomenon in *Hieracium pilosella* (Chapman *et al.* 2000 as *Pilosella officinarum*,

Chapman & Brown 2001, Krahulcová & Krahulec 2000), the mechanisms for the generation of variation in *H. pilosella* populations have not been widely addressed. The findings of the compatibility analysis indicate that sexual reproduction is of different importance in different populations. Possibly, this is related to both the frequency of sexual reproduction and the amount of seed recruitment occurring at these sites.

Although it is not possible to rule out effects of population founder differences, the level of population differentiation makes this explanation less likely. The high population variation observed and the evidence for the importance of sexual reproduction in determining population structure is something that must be considered when carrying out trials of potential control agents, whether chemical or biological. The generation of genetically complex population structure in populations with relatively low (1-3%) levels of sexual reproduction also has ramifications for the transfer of this trait to crop species. If the seemingly high frequency of mutation as detected by the compatibility analysis is associated with apomixis, this could also be problematic for the transfer of this trait to crop species.

Chapter IV. ENVIRONMENTAL INFLUENCES ON THE EXPRESSION OF APOMIXIS IN *HIERACIUM PILOSELLA*

IV.1.1 CORRELATION OF THE INCIDENCE OF APOMIXIS AT THE FIELD SITES WITH ENVIRONMENTAL PARAMETERS

Determining the extent of the environmental control of apomixis has several important implications for the transfer of this trait to economically important crop species (see Chapter I.2). The effect of environment, particularly daylength, has been shown to influence the expression of apomixis in some members of the Gramineae (see Chapter IV.2.), although this has not been reported in other groups. The correlation of differential expression of apomixis with varying environmental conditions may also provide an insight into the potential selection pressures for the breakdown of apomixis under field conditions. The identification of general cues, if any, will increase the understanding of the expression of apomixis in this species.

It is clear from chapter II.1 that there is variation in the frequency of sex in facultative apomictic *Hieracium pilosella* among both sites and years. This chapter aims to determine if it is possible to predict these shifts in the reproductive mode of *H. pilosella* using environmental parameters (described below) for the period of embryo sac formation to capitulum anthesis. A correlation of environmental conditions with reproductive mode will determine whether it is possible to partially predict the frequency of sexual reproduction under field conditions using environmental parameters. Density of rosettes will also be used as a possible predictor or reproductive mode (see below). Shifts in investment in seed production at the sites will also be examined over the environmental parameters. Several works have suggested a mixed mating system such as that found in *H. pilosella* is indicative of an optimal strategy for reproduction (Howard & Lively 1994, Lively & Howard 1995, Green & Noakes 1995), and it is of interest whether this will shift under different environmental regimes.

"Geographic parthenogenesis" and plasticity

The correlation of short-term changes in environment with shifts in reproductive mode allows the identification of the plasticity in the reproductive strategy of this species. While other works have examined the different distributions of apomictic and sexual individuals of the same taxa (see Mogie 1992, Asker & Jerling 1992 for reviews), the possibility that this pattern is also reflected in the variation in reproductive mode of facultative apomicts has not been tested. Morita (1976) examined the geographic distribution of different ploidy levels in *Taraxacum* spp. in Japan. Diploid plants of the several species examined occurred predominantly below 200m in altitude, restricted to warm areas that had a high degree of disturbance, although a few were found at up to 800m. In contrast, polyploids were restricted to arctic, sub-arctic, and cool temperate zones. It is known that diploid *Taraxacum* spp. are predominantly sexual, whilst polyploids are usually apomictic (Kirschner *et al.* 1994), indicating that the geographic distributions of Morita (1976) were concordant with reproductive mode. Gadella (1987) found that tetraploid sexual *Hieracium pilosella* was more common than pentaploid apomictic individuals at lower altitudes, and were also absent from Northern Europe. Both of these studies are typical of works investigating the geographic distributions of apomixis. The actual environmental conditions that are responsible for the disparities in distribution between apomicts and sexuals of the same taxa have so far not been identified.

Environmental parameters

The parameters investigated have been chosen based on the findings of other works examining the effect of the environment on apomixis. Although photoperiod has often been reported as being the only useful environmental parameter in predicting apomixis (see Chapter IV.2 for a review), some works have indicated that other parameters may also be of use. Both temperature and rainfall have been shown to be correlates of shifts in reproductive mode in *Dichanthium aristatum,* although the study that found this concluded that photoperiod was a more useful cue because it was more predictable (Knox 1967). This section will investigate the influence of altitude, mean daily temperature, rainfall and relative humidity on the expression of apomixis in *Hieracium pilosella.* The influence of photoperiod will be addressed in the next section (Chapter IV.2).

Ovule development and the environment

To determine the presence of any plasticity in the reproduction strategy of *Hieracium pilosella*, the frequency of sexual reproduction will be compared with the mean value of the environmental parameters for the seven days preceding pollination. This period has been chosen because it includes the critical time for ovule production. Koltunow *et al.* (1998) states that the initiation of meiosis occurs at stage 3 (following the description of Koltunow *et al.* 1998) of the development of the inflorescence, which coincides with approximately seven days before capitulum anthesis under field conditions (G. Houliston *pers obs.*). The timing of meiosis relative to the formation of an aposporous initial is thought to be the critical factor in the success of either embryo (Skalińska 1971, Koltunow *et al.* 1998). The rate of meiosis is possibly influenced by external environmental conditions, either directly, or following a general physiological shift in the plant metabolism. Using the environmental parameters for the period of megaspore formation and maturation allow the possibility of external conditions affecting the immediate environment and physiology of the ovule during this time. This gives the best possibility of identifying an influence of environment on the reproductive strategy of this species.

Density and reproductive plasticity

Density of the plants at the sites is also examined as a possible predictor of the frequency of sexual reproduction. Bishop and Davy (1985) found that there were differences in investment in reproduction and clonal growth at different rosette densities in populations of *Hieracium pilosella* in Great Britain. At moderate rosette densities (500 m^{-2} or lower), capitulum production was maximised, although the maximum production of apices was at high densities (~900 rosettes m^{-2}). It was also found that abortion of capitula was much more frequent at high densities, indicating a shift in reproductive investment to clonal growth under these conditions (Bishop & Davy 1985). The reasons for this shift in investment is not well understood. A change in strategy such as that observed by Bishop & Davy (1985) represents a substantial reproductive shift, and if capitula production in *H. pilosella* responses so strongly to density it is also possible that this is reflected in the levels of residual sex. This will also be investigated in this chapter. It is particularly interesting that there was not an increase in seed production with an increase in rosette density has as been observed in other taxa. This indicates a

non-linear response in reproductive mode to rosette density from *H. pilosella* (Bishop & Davy 1985).

The effects of the genetic variability on the fitness of progeny at different densities has been well studied (see Antonovics & Ellstrand 1984, 1985, Ellstrand & Antonovics 1985). Antonovics and Ellstrand (1985) concluded that the effects of genetic variation have a considerable impact on the success of dispersed seed progeny in *Anthoxanthum odoratum.* It is therefore of great interest if *H. pilosella* also possesses a plastic component in reproductive mode, as well as meristem initiation, in respect to density.

IV.1.2 MATERIALS AND METHODS

Sites and environmental parameters

The proportion of progeny produced by residual sexual events in facultative apomictic *Hieracium pilosella* was regressed against environmental factors to determine the influence of the environment on the reproductive mode of this species. Because the primary aim of this investigation is to determine the influence of the environment on facultative apomicts, and it has already been shown that obligate sexuality is genetically determined (Koltunow 1993, 2000, Bicknell *et al.* 2000), all crosses with obligate sexual plants were excluded from the data analysis.

Three of the field sites from chapter II.1. (Cass flat, Chilton valley and Little river) were chosen for this investigation due to their proximity to weather stations. Daily temperature, rainfall, and relative humidity means were recorded from these sites, and an average calculated for the week prior to each pollination event. Data were sourced from the Departments of Plant and Microbial Sciences (Cass flats site), and Geography (Chilton valley site) at the University of Canterbury, and the National Institute of Water and Atmosphere (N.I.W.A) (Little river site). Site and year were also recorded for analysis. Sites that were not adjacent to weather stations were added to the model, but only altitude, rank moisture and density of *Hieracium pilosella* rosettes per metre^2 were available as variates. Rank moisture was determined by subjectively ranking the

available moisture at each site from 1, the driest site, to 7 for the wettest, following examination of the site used. Details of the averages for temperature and rainfall for the week prior to each of the pollination dates can be seen in Appendix 5, Tables A5.1.1-A5.1.3.

Average climate conditions for the periods around the pollination events can be seen in Appendix 5, Tables A5.1.4 to A5.1.6. The summer of 1998-1999 was characterised in the five Waimakariri Basin sites by drought conditions, with rainfall in some areas as low as 50% of normal levels (Anonymous 1998). The Banks Peninsula site also had lower than normal rainfall, but this was still substantially more than the other sites (see Table A5.1.6). Cave stream experienced severe drought conditions with many inflorescences aborted and high mortality. Mean temperatures were of record levels in February, while January was the second warmest on record, dating back to 1853 (Anonymous 1999a, 1999b). December 1999 and January 2000 were wetter than the average, but dry conditions were prevalent in February (Anonymous 1999c, 2000a, 2000b). The summer of 2000/2001 was drier than average, and January was also cooler than the average (Anonymous 2000c, 2001a, 2001b).

Relatively large variations were observed in environmental factors over small spatial and temporal scales. The Chilton valley and Cass flats sites are approximately 900m apart, but due to the difference in altitude and aspect, there was considerable variation in the weekly mean of the three environmental variates even if the pollination date was the same (see Tables A5.1.1, A5.1.2).

Statistical analysis

Generalised Linear Model Analysis of Deviance was employed due to both the difference in predictor states, and the variation in replication. On some days at a single site, several samples were pollinated; and additionally, often an environmental factor, particularly rainfall, would not vary across several days. The binomial response was transformed using the arcsine transformation of Anscombe (1948) to normalise the response for gaussian GLM analysis.

Where:

$$p' = \text{arcsine} \sqrt{\frac{X + 0.375}{n + 0.75}}$$

Where: X is the number of positive results (i.e. the number of progeny sexual), and

n is the number of total results (total progeny)

Using this transformation of the data, each cross that resulted in at least one plant that survived until maturation was treated as an independent data point. The tables of frequencies presented in II.1.3 (Tables 2.1.5 a-c) show only the totals of hybrid (outcrossed) and apomicticly derived progeny produced at each site. For the analysis with environmental parameters the transformation took into account both the number of hybrid and apomictic individuals. Therefore a cross resulting in 70 progeny, 2 of which were hybrids, is scored correctly as being less facultatively sexual than one of 60 progeny and 2 hybrids. Although the tables in II.1.3 are useful to gain an overview of the frequencies of sex, the data following the transformation is more informative about the degree of facultative sexuality in each of the crosses.

All analysis was carried out using S-Plus Version 4.5 (Mathsoft 1998). The analysis was conducted using the Generalised Linear Model function, with the family type set as gaussian; using least squares estimation of the data distribution.

Climate sites

For the data from sites adjacent to the weather stations, analysis of deviance was structured with a block effect. Site (altitude) was the block effect, to control for potential genetic or environmental, other than those included, differences between the three populations. S-Plus interprets this correctly as long as the block effect is the first factor added to the hierarchical model. Although the experiment was also carried out over three seasons, this was not included as a factor as seasonal variation between years is primarily due to climate, and this is what was investigated with the variates. The continuous climate variates were temperature, rainfall and relative humidity. Slopes of partial regression between variates and the response, and significance levels, were then examined. The slope of the relationship indicates the nature of the response; whether

there is a positive or negative relationship between the two, and the magnitude of this relationship. Climate data for the 2000 / 01 season was not available for the Chilton valley site due to technical problems with the weather station. The sexual frequency data were excluded from the analysis after attempting to estimate the environmental parameters using data from the nearby Cass flats site. As the inclusion of the estimated parameters for the 2000 / 01 season made no difference to the results of the model the data were excluded from further analysis on the basis that information loss was minimal.

All sites

For all sites combined, the same arcsine transformed response was used in a generalised linear model including altitude (variate), rank moisture (variate) and average density of rosettes per m^2 (variate). Interpretation of the output was identical to that for the sites adjacent to weather stations. Rosettes per m^2 was determined by randomly sampling each of the field sites using a 250 mm x 250 mm quadrat, and counting the number of live, rooted, rosettes with at least 50% of their area within the quadrat. A total of sixteen quadrats were recorded per site, and the mean density per m^2 calculated from the mean of the replicates. Details of the values for each site can be seen in Table 4.1.1. Site descriptions can be seen in Appendix 1.1.

Table 4.1.1 Altitude, Rank Moisture and Densities of *Hieracium pilosella* rosettes, by site (± std error)

Site	Altitude (m)	Rank Moisture*	Density of rosettes (m^{-2})
Cave stream	700	1	507 ± 0.053 [641 ± 0.0362]†
Dracophyllum flat lower	790	3	598 ± 0.049 [919 ± 0.028]
Dracophyllum flat upper	810	2	1195 ± 0.042 [1206 ± 0.042]
Cass flats	580	4	390 ± 0.043 [440 ± 0.037]
Chilton valley	740	5	684 ± 0.033 [684 ± 0.033]
Little river	650	6	1561 ± 0.037 [1561 ± 0.037]

*Scored subjectively on available moisture, 1 – driest site, 7 – wettest site.
†All *Hieracium* spp. combined in brackets (Including *H. pilosella, H. praealtum, H.* x*stoloniflorum*).

All rosette densities were recorded only in the summer of 2002, and rank moisture was estimated using climate data for the regions in question.

Differences in seed production (total, filled and germinable) between sites and seasons is discussed in chapter II.1.

IV.1.3 RESULTS

In both analyses there was a significant relationship between at least one environmental variate and the level of sexual reproduction. This indicates that there was a significant effect of environment on the expression of apomixis in *Hieracium pilosella* under field conditions.

Due to the levels of replication in the experiment, the arcsine transformation of Anscombe (1948) was sufficient to normalise the distribution of the response variable for the analysis of the frequency of residual sexual reproduction. Generalised Linear Models were still applied for this analysis however, due to the variation in replication and the inclusion of factor and variate predictors. This also allowed the generation of partial regression coefficients to describe the relationship between variates and the response.

Climate sites

Analysis of Deviance from the generalised linear model elucidated the relationships between the environmental factors and the level of residual sexual reproduction. Significant relationships were detected between the level of residual sexual reproduction and the average values of environmental parameters for the week preceding pollination (Table 4.1.2).

There was a significant relationship between site and the level of sexual reproduction ($P = 0.0045$). This may indicate genetic differentiation among the sites, and a genetic component to the level of residual sexual reproduction at the sites (see Chapter III). Alternatively, this may reflect environmental factors that vary between the sites other

than those included in the analysis. The significance was found in the difference in reproductive mode between the Cass flats and Little river sites (see Table 4.13.). Of the environmental factors, temperature and rainfall were found to have significant effects (P = 0.0050, 0.0056, respectively), whereas relative humidity was not a good predictor (P = 0.3505). Analysing the slopes of the partial regression equations for both rainfall and temperature found positive, significant, relationships of both with the frequency of sexual reproduction (t-test; P = 0.0002, P = 0.0056, respectively). The slopes of both were found to be substantially different to zero, and explained a similar amount of variation in the analysis of deviance, although the slope of rainfall was steeper than that of temperature.

Table 4.1.2 Environmental Analysis, Climate sites 1998 – 2001.

Analysis of Deviance Table
Gaussian model
Response: Arcsine transformation of proportional data (Anscombe 1948).

	Df	Dev. Resid.	Df	Resid. Dev	F Value	Pr(>|F|)
NULL			189	0.3022810		
Site	2	0.01589091	187	0.2863900	5.570905	**0.0045**
Rel. humidity	1	0.00124938	186	0.2851407	0.875996	0.3505
Temp	1	0.01150761	185	0.2736331	8.068489	**0.0050**
Rain	1	0.01120458	184	0.2624285	7.856016	**0.0056**

Deviance Residuals:

Min	1Q	Median	3Q	Max
-0.047532	-0.027634	-0.008918	0.017228	0.131179

Coefficients:

	Value	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.0925838	0.05646649	-1.639624	
Site 1	-0.0121751	0.00432303	-2.816339	**0.0054**
Site 2	-0.0012058	0.00267085	-0.451481	0.6522
Rel. humidity	0.0014191	0.00050656	2.801521	**0.0056**
Temp	0.0063178	0.00167297	3.776376	**0.0002**
Rain	0.0160793	0.00573677	2.802859	**0.0056**

Null Deviance: 0.302281 on 189 degrees of freedom
Residual Deviance: 0.2624285 on 184 degrees of freedom

Table 4.1.3 Least significant difference test of Site as a factor for reproductive mode.

Sites that share a line beneath them are not significantly different at $\alpha = 0.05$.

Cass flats	Chilton valley	Little river
_____	_____	
	_____	_____

Partial residual plots for the variate predictors in the climate site analysis can be seen in Appendix 7.1,1. Tables of the correlation values between predictors can be seen in Appendix 7.1.2. Partial regression plots both give a graphical representation of the relationship between the variate and the response, and an indication of the goodness of fit of the model to the observations. Computationally, a partial residual plot is a plot of r*i* + bk X*i* k versus X*i* k [where r*i* is the ordinary residual for the *i*-th observation (ie response – fitted value from the model), X*i* k is the *i*-th observation of the k-th predictor and bk is the regression coefficient estimate for the k-th predictor (i.e. the regression coefficient (slope) multiplied by the value of the predictor)] (Mathsoft Inc. 1998). Therefore the slope of the plot describes the relationship between the predictor and the response, and the scatter of the points around the slope indicates the fit of the data to the model.

All sites

Generalised Linear Model Analysis of Deviance for the proportion of progeny produced sexually with altitude, rank moisture and density of *Hieracium pilosella* rosettes at the field sites as variates, can be seen in Table 4.1.4. A significant relationship was found between altitude and the level of residual sexual reproduction (P = 0.00597), although the slope of the partial regression line for this variate was not significantly different from zero (P = 0.54046), indicating that this is not an important linear predictor. Neither rank moisture or rosette density were found to have significant relationships with the level of residual sexual reproduction (P = 0.78547, P = 0.69663, respectively) (Table 4.1.4). No interaction terms were investigated due to variate nature and the differences in replication in the experiment.

Table 4.1.4 GLM Analysis of Deviance, all sites, Altitude, Rank Moisture and Density as predictors.

Analysis of Deviance Table
Gaussian model
Response: Arcsine transformation of proportional data (Anscombe 1948).

	Df	Dev Resid.	Df	Resid. Dev	F Value	Pr(>|F|)
NULL			289	21.98279		
Altitude	1	0.6325821	288	21.35021	7.673678	**0.006**
Moist	1	0.0061193	287	21.34409	0.074231	0.785
Density	1	0.0125551	286	21.33154	0.152303	0.697

Deviance Residuals:

Min	1Q	Median	3Q	Max
-0.3653155	-0.2241524	-0.07216265	0.133088	1.319378

Coefficients:

	Value	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.384929	1.58539	-0.242798	
Altitude	-0.001450	0.00237	-0.612857	0.540
Moist	0.017303	0.20141	0.085909	0.932
Density	-0.000123	0.00110	-0.112502	0.911

Null Deviance: 21.98279 on 289 degrees of freedom
Residual Deviance: 21.33154 on 286 degrees of freedom

Partial residual plots for the variate predictors for the analysis of all sites can be seen in Appendix 7.2.1. Tables of the correlation values between predictors can be seen in Appendix 7.2.2.

IV.1.4 DISCUSSION

The site effect in the climate sites analysis possibly indicates that a significant genetic component to the determination of the levels of residual sexual reproduction in *Hieracium pilosella* may be present (see also Chapter IV.3). Alternatively, there are important environment determinates that were not included in the analysis. A site effect could not be tested in the total data set due to use of variates (altitude, rank moisture and density of rosettes) to examine the possibility of a relationship between these and the

level of sexual reproduction, and the confounding of these with site. The interpretation of the site effect from the climate analysis as being due to genotypic differences between sites would seem to agree with the findings of Koltunow *et al.* (2001), that modifiers to the primary apomixis locus may be present and influencing the occurrence sexual embryos in *H. pilosella.* To find a site effect, the occurrence of environmental cues for the frequency of sexual reproduction other than those tested for in the "Climate site" analysis must vary between sites. Due to the relatively small spatial scale separating some of the sites these must fluctuate over relatively small distances if this is the case. For this reason, and that it has been shown that not only is there genetic differentiation between sites (Chapter II. 3, III), but that this can influence the reproductive mode (Chapter IV.3) the genetic explanation would appear more likely.

Climate sites

The relationship between the level of residual sexual reproduction, and temperature and rainfall indicates that there is an environmental aspect to the expression of apomixis, even once variation due to site is removed. This may underestimate the importance of these environmental factors, but is necessary due to the obvious strong genetic component for this trait (see also Chapter IV.3). That such a pattern was found when looking at environmental factors for the week preceding pollination indicates that there is a cue for sexual embryos in predominantly apomictic plants over this time frame. While it is possible that other time periods are also important, these were not able to be examined due to statistical limitations (mulitple hypothesis testing). Whether the positive relationships (Table 4.1.2) are proximate or causal is unknown, although it is possible that there is a physiological response in the plant to these parameters. An increase in either temperature or rainfall may effect the physiology of this species, favouring the sexual reproductive mode. It is possible that these changes in environment effect the surrounding physiological environment of the ovule, causing a change in the rates of cell division and ultimately the reproductive mode (Koltunow 1993). Such mechanisms, however, are speculative, as detailed study in this area has been lacking. Meiotic products in *Hieracium pilosella* are often displaced by apomictic initials due to the time it takes to complete meiosis (Skalińska 1971, Bicknell *et al.* 2000, Koltunow *et al.* 1998). It is possible that these environmental conditions accelerate production of a meiotic ovule at the expensive of the apomict by decreasing the difference in

development time for of meiotic and aposporous embryo sacs. It has been shown that *Hieracium* subgen. *Hieracium robustum* has more regular pollen meiosis at higher temperatures, indicating that temperature at least can play a role in the expression of meiosis in a predominantly apomictic species (Bergström 1969).

The lack of a relationship with relative humidity, and the very low amount of variation explained by this parameter, indicates that this is not a useful predictor of reproductive mode. It is worth noting that there was a relatively poor correlation between temperature, rainfall, and relative humidity (see Appendix 7.1.2) indicating that there was not a simple relationship between these parameters at the sites.

All sites

The relationship between the levels of residual sexual reproduction and altitude were significant, although the slope of the partial regression was not significantly different to zero (Table 4.1.4). This indicates either a very weak or no effect. The relationship was, however, slightly negative.

The lack of a relationship with rank moisture in this data set, and the significant relationship with rainfall for the climate sites, indicates that effects of water availability may be realised on short temporal scales, indicating a plastic rather than adaptive response. Alternatively, the moisture rank that was used may not be a good representation of moisture availibility at the sites.

The absence of a significant relationship with density is interesting in regard to the findings of Bishop and Davy (1985) and also Antonovics and Ellstrand (1985). The two sites (Little river & *Dracophyllum* flat lower) with the highest densities also had the highest proportion of progeny sexual. However this relationship was not found for all of the sites examined as some low density sites (e.g. Cass flats) also had relatively high frequencies of sex in at least one of the three seasons. That density can influence the initiation of inflorescences (Bishop & Davy 1985), but appears to be unrelated to the reproductive mode indicates that density effects are of lesser importance in the expression of apomixis, than they are for allocation of resources to growth (including vegetative propagation) or reproduction (i.e. seed production). It is interesting to note

that the ranges of density for the two studies overlap, with the densities at the sites in this study falling towards the median of those in Bishop and Davy (1985). It should also be noted, however, that the probability of initiating an apical meristem was greatest at medium densities in Bishop and Davy (1985). This study only examined whether a lineal relationship existed using inferential statistics, not whether this had a relationship in a factorial fashion as found by Bishop and Davy (1985). This was not possible in this study due to a lack of true replication of the density effect (i.e. confounded by site), excluding the possibility of meaningful statistics. That *H. pilosella* does not display reproductive plasticity in respect to density indicates that the interaction of dispersal and genetic variation in the progeny may not play as large a role in determining the fitness of this species as Antonovics and Ellstrand (1985) found for *Anthoxanthum odoratum*. Furthermore, this demonstrates that a strategy such as Ronsheim (1997) found for *Allium vineale*, whereby local adaptation of the progeny to the area closest to the parent favoured asexual reproduction, is unlikely in *H. pilosella*. If this were the case it would be expected that an increase in density, decreasing available substrate close to the parent, would favour sexually produced progeny, with a greater chance of survival outside the parental environment.

Site is significant as a factor in the climate analysis, but altitude is of marginal significance in the total data set as a variate. This indicates that the difference seen in the climate site analysis is most probably due to the genotype of the plants at the site, rather than a relationship with altitude. Although it therefore appears that a population level effect is more important, there is some indication, albeit weak, that the frequency of sexual reproduction decreases with increasing altitude. Further investigation would be required to determine if this is the case.

<u>The role of the environment on the reproductive mode of *Hieracium pilosella*</u>

The environment plays a significant role in the expression of apomixis in *Hieracium pilosella* growing under field conditions in New Zealand. Although it is clear that the difference between obligate sexuality and facultative apomixis is determined in a simple manner by genetic traits, the expression of facultative apomixis is more complex under field conditions. There is an influence of environmental factors on a very short temporal scale, covering the period of the initiation of the egg cell formation. This makes

prediction under field conditions difficult, and the occurrences of easily predictable environmental cues have not been identified. From the findings of this chapter, however, it is possible to conclude that seasons with higher temperatures and rainfall may result in higher levels of sexual reproduction in *H. pilosella.* The range of these parameters have been identified in this chapter, and it is possible by utilising the slope of the partial regression coefficients to predict the shift in investment in sexual reproduction by back-transforming the response to gain an indication of the proportion of sexually produced seed in question.

The presence of a relationship between rainfall and the level of sexual reproduction is most interesting from a practical viewpoint. In agricultural systems it is often possible to influence the available water to the plant by irrigation. In such situations it may be of benefit to seed producers to examine the performance of apomictic cultivars under different irrigation regimes during flower induction. This is of more interest than relationships with temperature as it is almost always impossible for this to be controlled in agricultural situations, perhaps with the exception of greenhouse crops. The findings of this chapter, however, strongly indicate that the identification of the correct genotype for apomixis for possible transfer to crop species is of more immediate concern than the management of the physical environment in which production occurs.

The lack of a density effect indicates that escape from the parental genotype (*sensu* the "Strawberry-Coral" model of Williams (1975)), is most likely not an important reason for the maintenance of sexual reproduction in this primarily apomictic species. The association with environmental parameters indicates a physiological mechanism at work, although the evolutionary selection pressure for this is unclear. The significantly greater number of progeny produced in crosses with at least one hybrid produced (Chapter II.1) indicates that the cost of sex may be offset by the increase in fecundity. This could reflect that the conditions associated with sexual reproduction are also conducive to growth and development, and that the two may not necessarily be causative. There are evolutionary reasons, however, why this may not be the case (see below). That both types are retained indicates an evolutionary optimal strategy, and also indicates that population demographics and structure may also have to be considered to identify the fitness advantage of this reproductive mode.

Recent work has shown that the expression of the oncogene *rolB* can considerably increase the initiation of aposporous initials in *Hieracium piloselloides* and also promotes meristem initiation (Koltunow *et al.* 2001). It is believed that the expression of *rolB* alters the cellular sensitivity to auxin, and that auxin may play a role in the initiation of floral structures in some species (Koltunow *et al.* 2001). As plant hormone production can be influenced by external environmental conditions, it is possible that the production of this or another hormone controls the production of meiotic embryo sacs. There may be control of optimum auxin production for the initiation of meiotic embryo sacs linked to the external cues identified, or a similar situation may exist for another phytohormone. Another group of phytohormones, gibberellins, have been shown to influence stem elongation but not flowering in some *Hieracium* subgen. *Pilosella* species (Petersen & Yeung 1972), so it is unlikely that these are responsible for controlling the expression of sex.

Fecundity and the frequency of sex

Of concern is the finding that the crosses resulting in at least one hybrid progenitor had significantly more progeny than those that were entirely apomictic (Chapter II.1.3), and that the higher levels of sexual reproduction were associated with conditions that would intuitively be associated with higher rates of growth. Both of these conclusions indicate that it is under the most favourable conditions that *Hieracium pilosella* reproduces sexually, and by maximising fecundity the cost of producing some progeny via sex is reduced as a proportion of reproductive effort. This is not surprising from an evolutionary perspective, but may be problematic when trying to both maximise yield and minimise recombination in transgenic crop stands (for an indication of the importance of outcrossed progeny in *H. pilosella* populations see Chapter III). That the majority of offspring are produced asexually, but that a small proportion are sexual under favourable conditions may, at first examination, best fit the framework of the "Tangled Bank" of Bell (1982). This model predicts that under conditions where the environment is becoming saturated, both a function of density and frequency, it will be beneficial for organisms to invest in sexual reproduction in the hope of producing progeny with slightly different resource requirements to their siblings. The lack of relationship with density, however, may make this interpretation questionable. Modern evolutionary works, often

by way of computer simulations, have attempted to identify optimal reproductive strategies in the face of mutation accumulation and parasite stress (Howard & Lively 1994, Lively & Howard 1994). In the case of *H. pilosella* there is little evidence of negative interactions with parasites due to the paucity of these in New Zealand. Other works have modelled the advantage of recombination in providing fitness increases in predominantly asexual populations through heterozygote advantage, and concluded a low frequency of sexual reproduction can maximise population heterogeneity (Green & Noakes 1995). This was contrasted by Peck and Waxman (2000), who produced a model that compared the fitness of asexual populations with and without a low level of sexual reproduction. As the obligate asexual population was modelled as being comprised entirely of heterozygotes, any low frequency of sex would cause a decrease in fitness in the model. For any benefits of sex to be realised, Peck and Waxman (2000), argue that it would have to be almost obligate. This is not the case in *H. pilosella* populations, and the situation observed is much more like that predicted by Green and Noakes (1995).

There is clearly some selection for the retention of a low level of sexual reproduction in field populations of *Hieracium pilosella.* This is evidenced by the presence of a genetic component to the mixed reproductive mode and the fact that variation in levels between populations can be observed. If sexual reproduction was selected against then it would be unlikely that individuals with higher rates of outcrossing would be maintained in the population through time. The complex reproductive phenotype of *H. pilosella* can therefore be attributed to both the genotypes of the plants and the environments in which it is found.

IV.2.1 THE ROLE OF PHOTOPERIOD ON THE EXPRESSION OF APOMIXIS IN *HIERACIUM PILOSELLA*

The among population variation observed in the frequency of sexual reproduction in facultative apomictic species has often been at least partially attributed to environmental effects (see Hussey *et al.* 1991, Asker & Jerling 1992, Mogie 1992). This has possibly been a result of the finding that apomictic individuals are commonly found at higher altitudes or latitudes than sexuals (see Chapter I.1.3); indicating that each type is better suited to a particular suite of environmental conditions. One of the most widely cited papers describing the role of the environment on the expression of apomixis (Knox 1967), even used a natural latitudinal gradient to investigate this phenomenon. This is also the rational behind several other investigations on this subject (see Evans & Knox 1970, Burton 1982). This chapter aims to determine if photoperiod, the most commonly cited environmental parameter in the control of apomixis (see below), is related to the frequency of sexual reproduction in *Hieracium pilosella.*

Geographic parthenogenesis

Numerous studies have discussed the role of the environment on the distribution and expression of apomixis (for example Nygren 1951, Knox & Heslop-Harrison 1963, Knox 1967, Evans & Knox 1969, McWilliam *et al.* 1970, Burton 1982, Bierzychudek 1989, Michaels & Bazzaz 1989, de Kovel & de Jong 1999, Koltunow *et al.* 2001). This has most often been prompted by the geographic distribution of apomicts, over ranges that would indicate that the environment is the key predictor in the distribution of such taxa. Why apomictic individuals of a taxon are often found at higher latitudes or altitudes than sexual individuals has inspired many studies, and almost as many theories, that attempt to resolve this question (see Turresson & Turresson 1960, Gadella 1972, 1987, Morita 1976, Asker & Jerling 1991, Mogie 1992).

Four main ideas as to why apomicts are commonly found at higher altitudes or latitudes have been outlined by Bierzychudek (1987b):

i) It has been suggested that pathogens and pests are less likely to be problem in high altitude or latitude areas (Bell 1982). Zoologists in particular have supported this theory (Bierzychudek 1987b). Citing the "Red Queen" hypothesis (van Valen 1973, Bell 1982,

1987), it would be expected that asexual individuals could persist in these areas whereas they may be excluded from more physically "benign" conditions, where it is expected that parasites will be more numerous. Lively (1992) found that asexual individuals of an aquatic gastropod, *Potomopyrgus antipodarum,* were restricted to deep-water habitats, where parasites were present in lower densities. This study may be able to be interpreted in regard to the "Red Queen" if it is assumed that the deep-water habitat is harsh for *P. antipodarum,* as it may well be in respect to search efficiency for food and mates. The finding that asexual individuals of *P. antipodarum* are restricted to these areas while the shallows where parasites are more numerous is populated with sexual individuals is compatible with this theory.

ii) Apomicts have better colonisation ability than sexual taxa, as they require a smaller initial founder population (Stebbins 1950). Botanists especially favour this theory (Marshall & Brown 1981, Asker & Jerling 1992, Holsinger 2000), however it does not necessarily explain why apomicts are predominantly found in harsher conditions. While it does predict that apomicts should be more common at the edges of a species distribution, this may not necessarily be harsh if the species is of recent arrival in the area.

iii) The offspring of sexual and apomictic individuals have been suggested to possess greater fitness than those with a combination of the two genotypes, and therefore an isolating mechanism has evolved to maintain the integrity of each (Lynch 1984). This is not a particularly powerful explanation, as many apomictic individuals can be found within otherwise sexual populations, and the resulting hybrids can exhibit comparable vigour to the parents. It should be noted, however, that very few taxa are known where apomixis can have intermediate expression to the point that sexually produced progeny approach 50% of the total progeny (see Knox 1967). This theory explains the possible reason for this lack of intermediate expression but does not explain geographic distributions. In the case of *Hieracium* subgen. *Pilosella,* there are several examples of hybrid taxa that are thought to arise from events between sexual and apomictic parents. *Hieracium* x*stoloniflorum,* a widespread, apomictic hybrid between *H. pilosella* and *H. aurantiacum,* is an example of such a complex (Webb *et al.* 1988). This taxon is also known from Europe, as well as several other hybrid complexes in the subgenus, which are also apomictic. These widespread hybrid species are of sexual origin, and it is

common for the putative parents to grow alongside their apomictic offspring (H. Chapman *pers comm*).

iv) Apomicts represent a "general purpose genotype" (Lynch 1984, Bierzychudek 1989, Michaels & Bazzaz 1989), as discussed in the previous section (Chapter IV.1.).

Photoperiod and apomixis

Environmental cues for the expression of apomixis have been investigated, mostly over natural environmental or latitudinal gradients. The findings of several papers investigating the influence of environmental factors on the reproduction of facultative apomicts have found that the proportion of seed produced via apomixis decreased as photoperiod increased (Knox & Heslop-Harrison 1963, Knox 1967 [*Dichanthium aristatum, D. annulatum, Calamagrostis purpurea*], Evans & Knox 1969 [*Themeda australis*]). Other works have been less conclusive or have found no predictability from this factor (McWilliam *et al.* 1970 [*Hyparrhenia hirta*], Burton 1982 [*Paspalum notalum*]). For a review of the studies on this phenomenon see chapter I.1.2. Knox (1967) found a relationship between temperature, and also rainfall, on the frequency of sex in facultative apomictic *D. aristatum,* although photoperiod was thought to be a better cue in this case as it followed a more predictable pattern. No other studies have reported a relationship between temperature or rainfall and residual sexual reproduction in facultative apomicts (although see Chapter IV.1).

This study aims to determine if photoperiod and temperature influence the expression of apomixis in *Hieracium pilosella.* Day-length and temperature will be manipulated to determine if any differences in the amount of apomictic and sexually produced seed is found.

Although studies of the effect of photoperiod on apomixis are lacking in the Asteraceae, de Kovel and de Jong (1999) did examine the effects of light intensity on morphological traits in diploid sexual and triploid apomictic *Taraxacum.* Apomicts were found to be more plastic for gross morphological traits, although this study was confounded by ploidy level. That apomicts can display a relatively high level of plasticity in morphological characters due to light intensity may indicate the potential importance of this as a general cue for these plants.

Previously, all studies of the effect of photoperiod on the expression of apomixis have used embryological examination to determine the percentage of progeny produced by each reproductive type (i.e. sexual or apomictic). This study will differ in that a heritable trait will be used as a marker for sexual events, following the artificial pollination of the plants in each of the treatments. The pollen donor in this case will be transformed by the insertion of a marker gene that can be phenotypically detected in the progeny.

Transgenic marker systems

The use of transgenic plants as a pollen donor in a known cross has potential for determining reproductive patterns in apomictic taxa. The insertion of a dominant gene with a known phenotypic expression that can be used as a marker for sexual events in facultative apomictic species has many advantages. The insertion character can be something that does not require the complete development of the progeny for detection, or even something that can be determined using a selective media such as an antibiotic. This allows faster progeny determination than conventional DNA or isozyme based methods. There is also a large cost advantage; and these two factors combined allow the screening of an otherwise unrealistic sample size in a short space of time.

Bicknell and Borst (1994) describe the transformation of a tetraploid, apomictic, accession of *Hieracium aurantiacum* by insertion of a gene for kanamycin resistance [neomycin phosphotransferase II, NPT II]. It was found that untransformed *H. aurantiacum* was highly susceptible to the antibiotic kanamycin, and therefore the transformed plants contained a distinct, heritable, character that would express itself in any hybridisation events, provided it was segregated to the progeny during recombination. Because only a single copy of the gene was inserted, it would be expected that only half of the sexually produced progeny would have this characteristic. The presence of a single copy only of the gene was determined by Southern analysis, which determined fragment lengths and expression of the insertions (Bicknell & Borst 1994). This same method has been applied to *H. praealtum* and *H. caespitosum*, using NPT II and other sequences (R.A. Bicknell *pers comm.*).

This method does have several disadvantages for general application. Firstly, the transformation is a time consuming and specialised procedure, and some species may be more amenable to successful transformation than others (R.A. Bicknell *pers comm.*). Secondly, there are regulations pertaining to the transformation of biological material, restricting experimentation to quarantine facilities. Finally, the results from the experiments require that frequencies must be multiplied by two to give an estimate of the actual number of sexually produced progeny. If sample sizes are relatively large this is not a concern, as the actual frequencies will approach the expected. This may be a problem if small sample sizes are desirable, or perfect resolution is required. When screening large numbers of progeny to determine general patterns this does not apply. In the case of smaller samples, DNA based techniques may be more appropriate. The use of a transgenic pollen donor has shown that it has worth as a screening method (Chapman & Bicknell 2000). Transformed individuals of *Hieracium praealtum* and *H. caespitosum* were used as pollen donors for wild-type individuals, and the progeny examined using a kanamycin positive media. It was found that the rate of hybridisation between *H. caespitosum* x *H. praealtum* was 1 % if *H. praealtum* was the paternal parent, and 0.5 % if *H. caespitosum* was used as the pollen donor. The frequency of sexual reproduction was calculated by multiplying the number of kanamycin resistant progeny by two and taking this as a proportion of the total progeny. This allowed the rapid characterisation of a relatively large number of progeny, and also demonstrated that this method is suitable for examining the reproductive patterns of facultative apomictic species (Chapman & Bicknell 2000).

The growth of untransformed *Hieracium aurantiacum* is inhibited at 5mg/L of kanamycin, and completely halted at 20mg/L. Transformed plants of *H. aurantiacum* could develop in up to 300mg/L of kanamycin, the highest level tested by Bicknell and Borst (1994). The level of kanamycin used in this study will be 50mg/L, the same level used by Chapman & Bicknell (2000) as an assay for this trait in *H. praealtum* and *H. caespitosum.* As this is two and a half times the level of total inhibition, this should prevent the occurrence of any false positive results.

IV.2.2 MATERIALS AND METHODS

Treatments

120 *Hieracium pilosella* ramets collected from the Chilton valley population (see Chapter II.1) were propagated under glasshouse conditions for six weeks until new growth had started to emerge. The plants were initially exposed to a photoperiod less than 10 hours to ensure no inflorescence initiation occurred, and then randomly assigned to one of six treatments in a factorial design with three levels of photoperiod, and two of temperature. The temperature treatments consisted of constant 24 hour, 30° C and 22° C treatments. The long photoperiod of 16 hours is greater than *H. pilosella* would experience under field conditions, although this is commonly used in the propagation of this species to induce rapid flowering (Yeung 1989) and seed germination (Makepeace 1985b). The 14 hour photoperiod is representative of mid-summer conditions and is know to induce flowering in *H. pilosella.* The 12 hour treatment approaches the minimum that is thought to trigger flowering in *H. pilosella* under glasshouse conditions (R.A. Bicknell *pers comm*). It should be noted that *Hieracium spp.* require exposure to at least five long days for flowering to be induced (Yeung 1989). The high temperature treatment (30° C) was carried out in Contherm Scientific plant growth chambers Cat. 620. All glasshouse lighting consisted of Phillips 50K S78 control units with 400watt SON 400 lamps, positioned 1.2m above the plants with a spacing of 1.5m between lights.

The early failure of the 30° C 12 and 16 hour treatments, and a shortage of plant growth chambers led to the abandonment of all the 30° C treatments. It was not possible to successfully control humidity levels in the growth chambers leading to high mortality and the failure of all flowering.

Pollination and screening of progeny

Once the plants in each of the other treatments had reached flowering they were pollinated with the transgenic kanamycin resistant pollen from a transformed accession of *Hieracium praealtum* (R4, Chapman & Bicknell 2000) following the method described in chapter II.1. The transfer of the NPT II sequence to *H. praealtum* was carried out using *Agrobacterium tumefaciens* strain LBA4404, carrying the binary vector pGA643, which contains the NPT II coding sequence, by the Crop & Food Research Ltd. Apomixis

group (Chapman & Bicknell 2000). As for the field crossing (Chapter II.1), two pollen donor capitula were used to pollinate each maternal parent to ensure no pollen limitation.

Germination of seed was as for the field-crossed plants (Chapter II.1) in respect to media and environmental conditions, but with the addition of 50mg/L of kanamycin to the agar solution after autoclaving. Plants that developed past the initiation of the cotyledons, and formed at least three true leaves, were deemed to be resistant, and therefore the product of sexual reproduction. 50mg/L is approximately 10 times the level of inhibition, and 2.5 times the level that totally halts development, of untransformed *Hieracium pilosella* and it is highly unlikely any non-resistant plants could survive at this level of the antibiotic (Chapman & Bicknell 2000).

As only a single copy of the NPT II sequence is inserted, giving a 50% underestimate of the rate of sexual reproduction, all frequencies of resistance were doubled before statistical analyses to estimate the actual levels of recombination.

Statistical analysis

The data were analysed using a single-factor analysis of variance, performed using S-PLUS Version 4.5 (Mathsoft 1998). The response (proportion of progeny resistant) was transformed using the arcsine transformation of Anscombe (1948) [see Chapter IV.1.2]. Day length was a factor in the ANOVA. All seed count data (total, filled and germinable) were also compared between the two day lengths using ANOVA, after applying a square root transformation to normalise the count data.

IV.2.3 RESULTS

The 12 hour photoperiod failed to induce flowering in all but a single individual in the 22° temperature treatment, and no germinable seed was produced (capitulum aborted). The 30° temperature treatment was found to be too harsh for plant survival, and the high humidity in the growth chambers resulted in the mortality of all plants from basal rot. No plants from the 30° C temperature treatment yielded any seed due to the complete

abortion of all capitula preceding the mortality of the plants. Both the 14 and 16 hour 22° treatments yielded viable seed from all of the putative crosses (Table 4.2.1).

Germination of the seed on nutrient agar proceeded as for the field study (Chapter II.1) until such time as the first true leaf emerged. Plants that did not contain the resistance gene for kanamycin would halt development, and the cotyledons would slowly lose pigmentation until mortality approximately four to six weeks after initial germination. Those plants that displayed resistance developed at a comparable rate to untransformed *H. pilosella* on agar without the antibiotic added. It was easy to identify these resistant plants among the remaining progeny (Figure 4.2.1). The time until development of the first three true leaves (and therefore meeting the requirement for resistance) was approximately six to eight weeks after initial germination.

The amount of seed production and proportion of seed produced sexually from the three treatments that initiated flowering can be seen in Table 4.2.1.

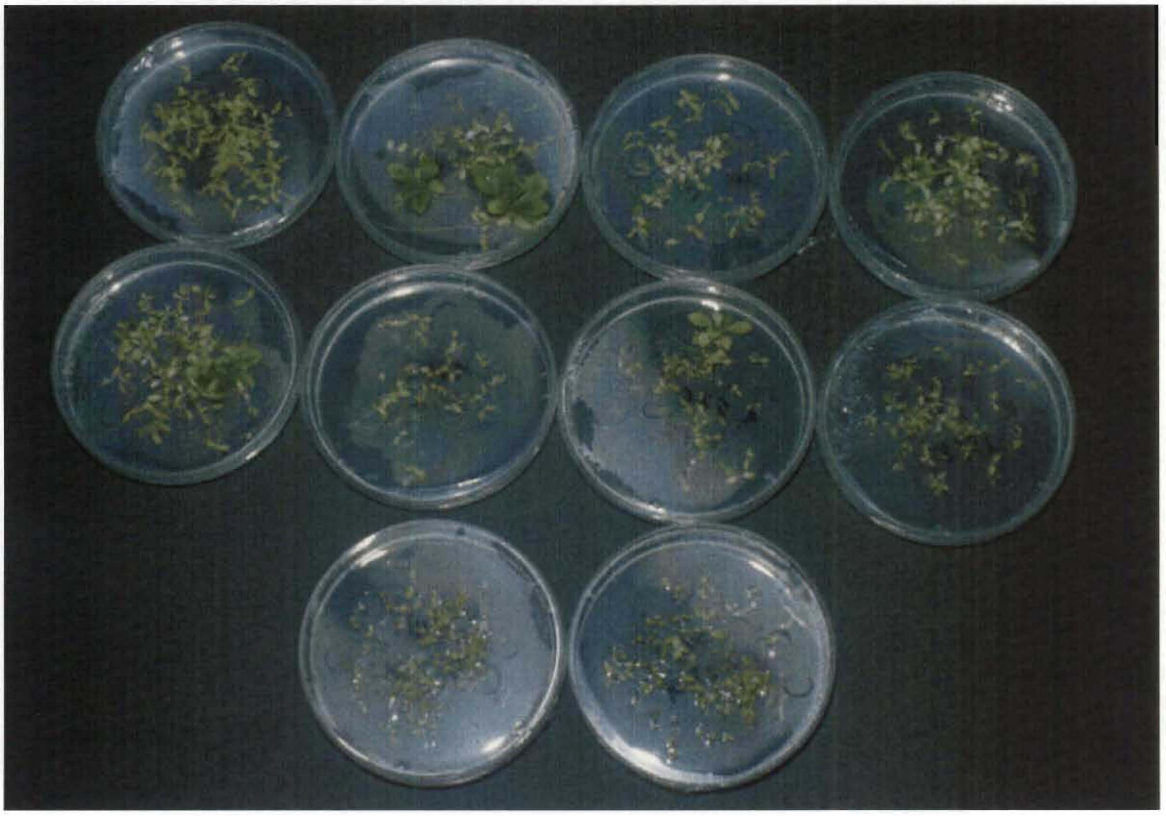
Table 4.2.1 Seed Production and Frequencies of Sexual Reproduction, 12, 14 & 16 hour photoperiods (± std error where indicated).

Treatment	Pollinations	Mean Total Seed	Mean Filled Seed	Mean Viable Seed	Total Progeny	No. Resistant*	Mean Resistant Seed
12 hour, 22°	1	121.00	30.00	0.00	0	0	0.00 (0.000 %)
14 hour, 22°	26	108.85 ± 0.141	58.27 ± 0.235	40.27 ± 0.394	1047	23	0.88 ± 1.442 (2.197 %)
16 hour, 22°	25	130.70 ± 0.135	46.63 ± 0.542	33.826 ± 0.510	778	10	0.435 ± 2.282 (1.285 %)

*Actual number of resistant progeny, multiplied by two prior to analysis.

Analysis of variance of the response (proportion of resistant progeny multiplied by two) for the two successful photoperiod treatments was carried out using an Arcsine transformation of count data (Anscombe 1948). Details of the ANOVA generated by S-Plus Version 4.5 (Mathsoft 1998) can be seen in Table 4.2.2. Although the percentage of progeny produced sexually under the 14 hour photoperiod was greater than the 16 hour treatment (Table 4.2.1), no significant difference in the rate of residual sexual reproduction was found between the two with an F test (P = 0.168) (Table 4.2.2.).

Figure 4.2.1 Screening for resistant (sexually produced) Progeny on a Kanamycin medium following the pollination of *Hieracium pilosella* with Kanamycin resistant *H. praealtum* (R4).



Resistant progeny can be clearly seen in petri dishes 2, 5 and 7 (numbered left to right). Non-resistant progeny are identified by their small stature and pale colour.

Table 4.2.2 ANOVA table of the proportion of sexual reproduction (Arcsine transformed) with 14 and 16 hour photoperiods.

	Df	Sum of Sq	Mean Sq	F Value	P (F)
Day length	1	0.00884	0.008844	1.9585	0.168
Residuals	47	0.21224	0.004516		

Square root transformation of seed count data and subsequent ANOVA found a significant difference in both total seed production and the amount of filled seed between the 14 and 16 hour treatments with an F-Test, although the significance for filled seed was only marginal. The amount of germinable seed was not found to be significantly different between the two treatments (Table 4.2.3).

Table 4.2.3 ANOVA – Total, Filled and Germinable seed production under 14 & 16 hour photoperiods. Square root transformation of count data.

Total seed

	Df	Sum of Sq	Mean Sq	F Value	P (F)
Day length	1	1.92834	11.9283	18.9145	**0.00007**
Residuals	50	32.1644	0.63068		

Filled seed

	Df	Sum of Sq	Mean Sq	F Value	P (F)
Day length	1	13.6323	13.6323	4.2630	**0.04**
Residuals	50	159.8898	3.19780		

Germinable seed

	Df	Sum of Sq	Mean Sq	F Value	P (F)
Day length	1	5.5683	5.56826	2.0205	0.17
Residuals	47	129.5241	2.75583		

The difference in sample size for the comparison between total seed production (n = 51) and germinable seed production and frequency of sexual reproduction (n = 48) was due to infection of agar plates leading to the loss of three samples. Infection of plates was not as much of a problem as in the field study (see Chapter II.1) due to all crosses being

carried out under glasshouse conditions which reduces the occurrence of fungi in the fruit wall (R.A. Bicknell *pers comm.*). The addition of the antibiotic to the media also inhibits fungal growth.

IV.2.4 DISCUSSION

Photoperiod and the frequency of sex

Under both 14 and 16 hour photoperiods, the Chilton valley population had a low frequency of residual sexual reproduction. Although considerable variation was observed between the frequency of sexual reproduction in the two treatments, with a higher percentage of progeny under a 14 hour photoperiod sexual, the difference was not found to be statistically significant ($P = 0.168$). This would indicate that the expression of apomixis in this species is not correlated with photoperiod, as has been found in other species. This finding is interesting in the context of other studies.

Although there is an effect on reproductive investment in this species with respect to photoperiod (total and filled seed production), this is not reflected in the levels of residual sexuality. This indicates that photoperiod is not an important cue for investment in sexual reproduction in *Hieracium pilosella.*

Although no significant effect of photoperiod on the reproductive mode of *Hieracium pilosella* was observed, the trend in the data was for the frequency of sex decreasing as photoperiod increased. Studies in the Gramineae have found that where there is an effect of photoperiod, the amount of sexual reproduction increases as photoperiod increases (Nygren 1951, Knox & Heslop-Harrison 1963, Knox 1967, Evans & Knox 1969, McWilliam *et al.* 1970, Burton 1982). The difference in reproductive strategy in these species may be related to the different mechanisms for apomixis in these groups, although both are in the category of aposporous apomicts.

The inclusion of only a single population of *Hieracium pilosella,* and the use of only two photoperiods may have limited the ability to test for a general relationship between

photoperiod and the frequency of sexual reproduction. The population selected (prior to having knowledge of field frequencies of sexual reproduction - see Chapter II.1) produced only a relatively low proportion of progeny sexually, also reducing the ability to test for an effect. However, it is possible to conclude that the pattern observed in the Gramineae is not found in *H. pilosella*, as if anything the frequency of sex decreased rather than increased under longer photoperiod.

Photoperiod and seed production

The total reproductive output of *Hieracium pilosella* from the Chilton valley site shifted under 14 and 16 hour photoperiods. Total and filled seed production was significantly different under 14 and 16 hour photoperiods, with an increase observed in total seed production , and a decrease in filled seed production, under 16 hour light. This difference was not observed in the amount of germinable seed, which was not found to be significantly different between the two treatments. This would indicate that although more seed were initially produced, these were not viable, possibly due to genetic effects such as the disruption of parthenogenesis. The reason for such a change in reproductive investment under the two different photoperiods is unknown. Other works have either not reported or not investigated any effect on total or germinable seed output. As most studies have been in the Gramineae, on species which produce numerous seeds, most have sub-sampled an inflorescence for cytological investigation and not considered seed number or viability (see Nygren 1951, Knox & Heslop-Harrison 1963, Knox 1967, Evans & Knox 1969, McWilliam *et al.* 1970, Burton 1982). Further investigation would be necessary to fully identify the mechanism behind this pattern. Given that the experiment was conducted at fertiliser and water levels much higher than would be commonly experienced in the field, it is unlikely that these factors are as limiting as under field conditions.

That the higher level of sexual reproduction was associated with the higher amount of germinable seed, although neither being significantly so (see also Chapters IV.3, II.1), indicates a physiological mechanism at work. The loss of germination rate with the increase in seed production is interesting, and this may have consequences for seed production at the population level over the growing season. The inclusion in this comparison of only one population also decreases the ability to test this idea. Further

investigation should include numerous populations, and also night interruption experiments, to control for the effects of total light energy. As Knox and Heslop-Harrison (1963) point out, the control of this phenomenon may not be strictly photoperiod *per se,* as in all cases the total amount of light energy received by the groups differs. The loss of the temperature treatments due to the failure of the growth chambers also removed the possibility of finding are interaction between temperature and photoperiod. In respect to the findings of chapter IV.1, a test of controlled temperature levels may be worthy of further investigation.

Kanamycin resistance as a marker for sex

The transgenic marker system employed in this study was found to be a convenient method to determine frequencies of sexual reproduction in facultative apomicts. As found by Chapman and Bicknell (2000), resistant individuals were very easy to identify on a kanamycin positive media, eliminating the requirement for conventional progeny testing. Frequencies of sexual reproduction found in this study were comparable to the levels in other glasshouse trials (see Chapter IV.3), indicating that this provides a suitable measure for between population comparisons. The possibility of different efficiencies of pollen sources still exists, but for this trial where only differences between treatments are investigated, this is not a concern. That no obvious difference was found in the frequency of sexual reproduction between methods (transgenic vs morphological marker systems – see Chapter IV.3), also indicates that the necessity to double the frequencies of sexual reproduction observed to obtain an estimate of the actual maternal potential for sexual reproduction is not a concern. This method was found to be much less labour intensive, and also considerably faster, than growing plants through to maturity to identify hybrids by gross morphology, or even flow cytometry. That a known heritable trait is also used for screening makes this an elegant method for the detection of hybrid progeny.

For the practical purposes of this work, it is not possible to manipulate photoperiod under field conditions. Although the knowledge of the importance of this may increase the understanding of apomixis in *Hieracium pilosella,* it is of limited direct application to apomixis and bio-control programmes.

IV.3.1 QUANTIFYING THE EFFECT OF GENOTYPE AND ENVIRONMENT: A COMMON GARDEN EXPERIMENT.

A fundamental tenet of biology is that the phenotype of an organism is a product of both the genotype and the environment. This chapter specifically aims to isolate the effect of the environment on the reproductive phenotype of the facultative apomict, *Hieracium pilosella* by comparing reproductive performance under both field and glasshouse conditions. This will also allow the influence of population genotypic variation on the expression of residual sexual reproduction to be measured by determining whether the influence of the environment on this trait is dependent on the population from which the samples are taken.

Plasticity in reproductive mode and the commercial application of apomixis

If the expression of apomixis in *Hieracium pilosella* is indeed a plastic trait, the role of the environment on the expression of this has important implications for its use in agriculture. Although some degree of residual sexual reproduction is desirable to allow the production of new genotypes (Koltunow *et al.* 1995), too high a level will result in populations with an unacceptable amount of heterogeneity for commercial purposes (Hanna 1995, Hanna & Bashaw 1987). It is imperative to quantify the levels of plasticity in this trait to increase the understanding of how this could potentially influence the structure of crop stands.

Field based studies will display the range of a trait only under the conditions prevailing in the field during the study. If the variation present in a trait is determined considerably by the environment (i.e. is phenotypically plastic), then the full range of this trait may not necessarily be observed under normal environmental conditions.

The common garden experiment

Common garden experiments have been widely used in the study of apomictic taxa, particularly to investigate the effect environment has on the breeding system or competitive abilities of individuals from different sites (see O'Connell & Eckert 2001, de Kovel & de Jong 1999, 2000, Michaels & Bazzaz 1989, Bierzychudek 1989). These studies almost without exception compare apomictic and sexual plants of the same taxa.

Bierzychudek (1989) and Michaels and Bazzaz (1989) carried out such experiments using both sexual and apomictic accessions of *Antennaria* spp. Both these studies involved individuals from different areas being grown in one location, under varying environmental conditions, and the survival and biomass recorded over artificial environmental gradients. Both studies found that the apomicts possessed greater environmental tolerance, and in the case of Bierzychudek (1989) the apomict was superior under all environments (see Chapter I.1.3 for a discussion of this work). Few studies of this nature, with the exceptions of Ceplitis (2001) looking at the shift between seed and bulbil production in *Allium* spp. and O'Connell & Eckert (2001) examining reproductive effort in *Antennaria parlinii,* have investigated whether reproductive mode differs with environmental conditions. Johansson (1994) examined the difference between clones at the margins and centres of *Ranunculus lingua* populations, albeit under field conditions, and found there was a distinct difference in the reproductive investment of the two groups. Those at the centre of populations seemed to be under greater competitive and parasite stress, and as a result produced a few large rhizomes. At the margins, density-independent mortality controlled population density, and plants produced many small rhizomes. This study is unique in that it includes a clonal organism, and investigated the reproductive investment of ramets under different environmental conditions.

This chapter will use a combination of field and glasshouse data to quantify the influence of genotype and environment on reproductive mode. This is in contrast to the majority of common-garden experiments, which primarily look at biomass production, root to shoot ratio (see Willis & Blossey 1999, Neuffer & Eschner 1995), or susceptibility to pests or diseases (see Roy 1998, also Chapter V).

Common garden experiments are often employed in the place of reciprocal transplant experiments, as they offer both a higher degree of control and simpler logistics than field based studies (Bierzychudek 1989, Neuffer & Eschner 1995, Willis & Blossey 1999). They also avoid the most common problem with reciprocal transplant experiments, density effects, which often confound the effect of treatments (Roy 1998). A disadvantage of such an approach is that the different performance of organisms from two areas when grown under uniform conditions is often assumed to be due to genetic

differences between the two groups, but rarely is this actually confirmed (Briggs & Walters 1997). This study avoids this problem by growing individual plants from single rosettes collected in the field through to flowering under glasshouse conditions. It is unlikely the environmental conditions of the field sites would therefore influence the reproductive output of these individuals.

It is clear from chapters II.1 and IV.1 that there are significant differences between the levels of sexual reproduction at the field sites included in this study. A common garden experiment lends itself to problems such as this as it allows the separation of genetic and environmental effects. By recording the levels of sexual reproduction under both field and glasshouse conditions, it will be possible to determine the relative influence of genotype and environment on the levels of sex. While comparing levels of sexual reproduction at sites with different environmental conditions in the field may indicate genetic differences (see Chapter IV.1), it is necessary to grow plants under controlled conditions, ideally from an early developmental stage, to actually test the magnitude of this effect.

IV.3.2. MATERIALS AND METHODS

Sampling

Plants from the three most central Waimakariri basin sites (Cass flats, Chilton valley & *Dracophyllum* flat lower) were collected in late 1999 and grown under uniform conditions until spring 2000. The three samples were chosen to represent a continuum from almost obligate apomixis, to a sample containing some individuals that were most likely obligate sexuals. The frequencies of sexual reproduction at the sites used (% sexually produced progeny) for the 1999 –2001 seasons were as follows: Cass flats (6.54, 0.65, 2.72), Chilton valley (1.31, 0.63, 1.22), *Dracophyllum* flat lower (21.57, 100.00, 38.16) (see Chapter II.1). This provided the maximum possible resolution between the possible genotypic and environmental influences on the expression of sexuality in this species, at the population level. The relative contribution of genotype and environment at the individual level is addressed in chapter V.

Experimental design

The plants were propagated under glasshouse conditions over the winter of 2001, and flowering was initiated using high-pressure sodium vapour lights with a 16 hour photoperiod. The buds were then covered following the method of the field crossing experiment (Chapter II.1.2). Seventeen individuals of *Hieracium pilosella* from each of the Cass flats and Chilton valley populations, and fifteen from *Dracophyllum* flat lower, were hand pollinated with *H. aurantiacum.* The propagation of seed and progeny, and the analysis of key morphological traits that would indicate hybrid origin (and therefore sexual reproduction) was also carried out following the method of chapter II.1.

Frequencies of sexual reproduction recorded from the glasshouse populations were compared to those at the respective field sites (see Chapter II.1), to determine if the change in environmental conditions had any influence on reproductive mode. By comparing both within and between populations it was possible to draw conclusions on the nature of any plasticity in reproductive mode. Total, filled and germinable seed production was also recorded and compared among sites and environments. This allowed identification of shifts in reproductive strategy under different environmental conditions for the three sites examined.

Statistical analysis

All comparison was by way of ANOVA, including the frequencies of sexual reproduction, and seed production, under both field and glasshouse conditions. The frequency of sexual reproduction was Arcsine transformed following the method of Anscombe (1948) (see Chapter IV.1) to remove the underlying binomial distribution. Two factors were examined, site (Cass flats and Chilton valley) and environment (field and glasshouse), as well as the interaction term between the two. The *Dracophyllum* flat lower site was excluded from the ANOVA after it was found that all individuals collected from this site were obligate sexuals. Although three years of data were included in the data from the field populations, season was not included as a block effect due to this not being replicated in the glasshouse.

Seed production for the Cass flats and Chilton valley sites was also analysed using ANOVA, but the response in this case was square root transformed to remove the underlying Poisson distribution (Howell 1992, Zar 1996). Total, filled, and germinable seed production were used as responses, with the site and environment (and the interaction of the two) as factors. Only the first two years of the field data were used in the analysis and this coincided with the time frame of the glasshouse investigation.

Relative DNA ploidy equivalents of the plants from the Cass flats and Chilton valley sites were recorded using flow cytometry, following the method of chapter II.3. This was both to further investigate if ploidy level influenced residual sexual reproduction, and to confirm the difference observed in ploidy level between these two sites from the field study (Chapter II.3).

IV.3.3. RESULTS

Frequencies of sex

The *Dracophyllum* flat lower site was comprised of entirely obligate sexual individuals, and for this reason the results from this site were excluded from further comparisons, although the frequencies of sexual reproduction for this site can be seen in Table 4.3.1. The two other sites were found to produce primarily apomicticly derived progeny, under both field and glasshouse conditions.

The amount of sexual reproduction was significantly different between the glasshouse and field environments. Site (population) and environment were both found to significantly influence the amount of sexual reproduction, as can be seen in Table 4.3.2, ($P = 0.002$ and $P = 0.02$ respectively). Site explained the greatest amount of variation, indicating that the genetic structure of the population may be more important than environment in determining the reproductive mode of *Hieracium pilosella*. The interaction term between site and environment was not significant ($P = 0.81$) (Table 4.3.2).

Table 4.3.1 Frequencies of sexual reproduction, Cass flats, Chilton valley, *Dracophyllum* flat lower; field and glasshouse results (± std error).

Site	Treatment	No. flowering	No. Sexual	No. Crosses	Mean progeny per cross	% Sexual
Cass flats	Field 98/99	585	35	18	32.50 ± 0.597	5.98
	Field 99/00	155	1	13	11.92 ± 1.143	0.65
	Field 00/01	676	17	17	19.00 ± 0.986	2.51
	Glasshouse	863	34	17	50.76 ± 0.423	3.94
	All treatments	**2279**	**87**	**65**	**35.06 ± 0.657**	**3.82**
Chilton valley	Field 98/99	794	5	22	36.09 ± 0.517	0.63
	Field 99/00	535	7	21	25.48 ± 0.738	1.31
	Field 00/01	984	12	21	39.77 ± 0.501	1.22
	Glasshouse	549	17	17	32.29 ± 0.420	3.10
	All treatments	**2862**	**41**	**81**	**35.33 ± 0.588**	**1.43**
Draco flat lower	Field 98/99	408	88	8	51.00 ± 0.310	21.57
	Field 99/00	8	8	2	4.00 ± 0.707	100.00
	Field 00/01	152	58	8	46.86 ± 0.527	38.16
	Glasshouse	1237	1237	15	82.47 ± 0.468	100.00
	All treatments	**1805**	**1391**	**33**	**52.52 ± 0.781**	**77.06**

Table 4.3.2. ANOVA of Sexual Reproduction versus site (Cass flats & Chilton valley) and environment (Field & Glasshouse).

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	1	0.0247525	0.024753	10.428	**0.002**
Env	1	0.0128495	0.012850	5.413	**0.02**
Site: Env	1	0.0001349	0.000135	0.057	0.8
Residuals	104	0.2468640	0.002374		

Seed production

Viable seed production from the obligate sexual plants from the *Dracophyllum* flat lower site was higher than the Cass flats and Chilton valley (facultative apomictic) sites under glasshouse conditions, although the difference was not as great in the field. The *Dracophyllum* flat lower site also exhibited greater variation in seed production than the other sites under field conditions. Two of the field seasons (98 / 99 and 00 / 01) had a

considerably higher average number of progeny per cross than the sites comprised of apomictic individuals, and the remaining season (99 / 00) producing a substantially lower average (Table 4.3.1).

Experimental crosses with the facultative apomictic plants under glasshouse conditions resulted in a significantly higher number of progeny per cross than in the field (Table 4.3.1), indicating a significant impact of the environment on the reproductive output of this species. Total seed production per cross was also found to be significantly influenced by the environment, with more seed set under glasshouse conditions (Table 4.3.1). This was not found to be the case for the number of filled or germinable seed per cross, however. For total, filled and germinable seed production there was a significant interaction term between site and environment (Table 4.3.1).

Table 4.3.3 Seed production, glasshouse and field conditions

Total seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	1	0.90707	0.90707	1.27246	0.26
Env	1	57.16413	57.16413	80.19153	**0.0000001**
Site:Env	1	5.64606	5.64606	7.92047	**0.006**
Residuals	104	74.13587	0.71284		

Filled seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	1	5.3320	5.33199	2.435493	0.12
Env	1	2.7722	2.77225	1.266280	0.26
Site:Env	1	13.5967	13.59666	6.210550	**0.01**
Residuals	104	227.6856	2.18929		

Germinable seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Site	1	0.1311	0.13112	0.034375	0.85
Env	1	13.8467	13.84668	3.630213	0.06
Site:Env	1	23.4127	23.41273	6.138164	**0.02**
Residuals	104	396.6861	3.81429		

The seed production at of each site, irrespective of environment, and the production by environment, irrespective of site, can be seen in Table 4.3.4. The amount of seed produced at each site under the two different environmental treatments can be seen in Table 4.3.5. The significance of the interaction term in each of the analyses of seed production can be seen from the means for each treatment in this Table (4.3.5). The increase in seed production in the glasshouse for each of the categories (total, filled and germinable) was much greater for the Cass flats than the Chilton valley site (Table 4.3.5).

Table 4.3.4 Seed production under field and glasshouse conditions by site and environment (All values ± std error).

	Site		Environment	
	Cass flats	Chilton valley	Glasshouse	Field
Total seed production	97.08 ± 0.243	91.39 ± 0.216	115.47 ± 0.143	86.33 ± 0.205
Filled seed production	45.48 ± 0.527	53.33 ± 0.438	51.12 ± 0.325	49.55 ± 0.526
Germinable seed production	31.32 ± 0.849	31.88 ± 0.713	42.97 ± 0.490	27.69 ± 0.878

Table 4.3.5 Seed production of Cass flats & Chilton valley sites under field and glasshouse conditions. (All values ± std error).

	Glasshouse		Field	
	Cass flats	Chilton valley	Cass flats	Chilton valley
Total seed production	123.47 ± 0.126	107.47 ± 0.128	85.72 ± 0.198	83.50 ± 0.212
Filled seed production	56.59 ± 0.371	45.65 ± 0.176	36.13 ± 0.580	49.63 ± 0.459
Germinable seed production	52.59 ± 0.435	33.35 ± 0.420	14.23 ± 1.014	23.84 ± 0.780

The percentage of filled and germinable seed of the total seed production for the three sites can be seen in Appendix 8. The significant interaction term between site and environment for total, filled and germinable seed production, indicates a difference in response to the change in environment for the two populations.

The nuclear DNA content of the plants at the Cass flats and Chilton valley sites were highly significantly different from each other in those plants of approximately pentaploid level. The standard error of the values for DNA ploidy equivalent at both of the sites was low, resulting in the highly significant difference (Table 4.3.6). At both sites a single tetraploid individual was also recorded.

Table 4.3.6 Comparison of DNA Ploidy equivalents of the pentaploid states (Standard Two-Sample t-Test), Cass flats and Chilton valley sites (± Standard Error).

Site	DNA ploidy equivalents	t-Test
Cass flats	45.420 ± 0.00649	t = 11.6476,
Chilton valley	46.594 ± 0.00591	df = 30
		p-value < 10^{-12}

IV.3.4 DISCUSSION

Sexual reproduction

The significant effect of environment (P = 0.02), and a highly significant effect of site (P = 0.002), on the expression of sexual reproduction in *Hieracium pilosella* indicates a strong genetic component, and a moderate environmental component, to reproductive mode. Frequencies of residual sexual reproduction were much more similar between the two populations comprised of facultative apomictic individuals under glasshouse than field conditions. The insignificant interaction term indicates that neither population showed a greater response to the change in environment than the other, indicating a universal response to the change in conditions. Therefore, despite the differentiation between the sites as shown by the site effect (see also Chapter III), both responded in a similar manner to the more benign glasshouse conditions. Over both environments, the Cass flats site had a significantly higher proportion of progeny produced sexually, despite the lower frequency for the 1999 / 2000 field season. The lower frequency of sexual reproduction during that season is possibly due to sampling error, with the extreme drought conditions of that year resulting in very low sample sizes.

Seed production

The total seed production of the two sites was not found to be significantly different, although total seed production between the two environments was highly significant (P = 0.000001). The interaction effect between site and environment for total seed production was also significant (P = 0.006), with the Cass flats population having a greater increase in total seed production than the Chilton valley population under glasshouse conditions. Although no significant effect of site or environment was found for filled seed production, the interaction term was moderately significant (P = 0.014), again with the Cass flats population showing a greater response to glasshouse conditions than Chilton valley. The same pattern was reflected for germinable seed (P = 0.015), again with the Cass flats population responding to environment to a greater degree than Chilton valley. These findings indicate that both the environment, and the genotype of the plants, plays an important role in the reproductive strategy of this species. The significant interaction terms for total, filled and germinable seeds indicates that the Cass flats population is much more plastic in seed production than Chilton valley, although the environment is also important in total seed production. As has been found for the levels of residual sexual reproduction, genotype (as shown by "site" effects – Chapters IV.1, IV.2) is often more important than plasticity in determining the reproductive mode of this species.

Nuclear DNA content variation

Further evidence for the genetic control of residual sexual reproduction in *Hieracium pilosella* is the significant difference in DNA ploidy equivalents recorded between the sites (see also Chapters II.3, III). That genetic differences at this level can be detected between sites indicates that it is also likely that there is divergence in other traits. The genetic component to the reproductive mode may be one such trait with among-population divergence. A genetic mechanism for such among-population differentiation in the rates of apomixis in *H. pilosella* has also been postulated (Koltunow 2000).

The values of the DNA ploidy equivalents from this study are comparable to the values recorded in chapter II.3, especially for the Cass flats site. The Chilton valley site has a slightly lower mean than recorded in the study of the plants grown from seed, although the standard error from the seed progeny is greater. It would seem that this is most likely

due to sampling effects as the value of the Cass flats plants did not vary over the two studies, as would be expected if this was an instrumentation problem.

Evolutionary considerations

The Cass flats site on average also possessed a greater range of variation in residual sexual reproduction than the Chilton valley site. The association of plasticity in these two traits is interesting when considering the reproductive strategy of this species. As the higher rates of residual sexual reproduction at the Cass flat site was associated with seasons with higher seed production (see Table 4.3.1), there may be an evolutionary strategy to this reproductive mode at this site. If sexual reproduction is more prevalent in conditions of high seed output (see also Chapter II.1) this may indicate an avoidance of intra-specific competition by avoiding direct conflict with siblings due to the possession of the exact same requirements (the "Tangled Bank" model *sensu* Bell 1982).

It is interesting to note that the average amount of progeny per cross for the obligate sexual site is almost exactly twice that of the apomicts. This is particularly interesting in respect to the maintenance of sex question. Although the amount of seed production for these plants under field conditions in some years is much lower than the apomicts, under the more favourable conditions of the glasshouse there is the potential for much greater seed set, to the level that would offset any cost of sex. This may indicate a change in reproductive strategy with reproductive type, as has been suggested by de Kovel and de Jong (2000). The sexual type may represent a genotype that is able to become specialised to particular conditions, and therefore occupying a different niche to the more "general-purpose" apomict (de Kovel & de Jong 2000). It is also possible to explain this in the framework of Ceplitis (2001) who suggests that different reproductive modes may be favoured in different seasons; the magnitude of variation in fecundity for these plants as observed in the field among years indicates that there is potential for this to operate. This finding is particularly interesting when compared to that of Gadella (1991a), who found that pentaploid apomicts produced slightly more germinable seed than tetraploid sexuals under experimental conditions. The influence of environment on the fecundity of *Hieracium pilosella* is large, and it is also clear that between population differences exist, as has been found for reproductive mode. This is further evidence for the differentiation

of populations and evolution at the population level occurring in *H. pilosella* in this country.

Chapter V. GENOTYPIC VERSUS PLASTIC RESPONSES OF *HIERACIUM PILOSELLA* TO BIOTIC AND ENVIRONMENTAL STRESSES.

V.1.1. INTRODUCTION

It has been suggested that the frequency of sexual reproduction in facultative apomictic *Hieracium* subgenus *Pilosella* is determined by genotype, particularly the presence of modifiers to the primary apomixis locus (Koltunow 2000, Bicknell *et al.* 2001). Other works, however, have attributed variation observed in the frequencies of sex to environmental sources (Hussey *et al.* 1991, Mogie 1992, Asker & Jerling 1992), and there is evidence in some groups that this is the case (for a review see Chapter IV.2). This chapter aims to determine the influence of both genotype, and the biotic and nutritional environment, on the reproductive mode of *H. pilosella*. Transgenic accessions of *Hieracium* subgen. *Pilosella* are used as pollen donors, with kanamycin resistance as a marker for recombination (see Chapter IV.2).

Genotypic control of apomixis

The effect of genotype on the levels of residual sexual reproduction is a critical factor for the future transfer of genetic material for apomixis from *Hieracium* subgen. *Pilosella* species to crop species. Five genotypes are chosen to represent a range of frequencies of sex as determined from the results of the field experiment (Chapter II.1). Each genotype is replicated in each of the treatments. As has been shown in chapters IV.1 and IV.3, genotype may play an important role in determining the reproductive pattern of *H. pilosella*. This design will allow control over genetic factors by limiting variation to only the five selected genotypes, making it possible to determine if a directional response can be found universally over the genotypes. This will test whether the response of *H. pilosella* can be generalised to specific factors, or whether there are differential directional responses to external cues by the different genotypes. This will give a measure of the predictability of the system.

Biotic factors

Biotic factors have been postulated as a main selective pressure for the maintenance of sexual reproduction. This is also a possible factor in the field as there are two known species of potentially pathogenic fungi present on New Zealand populations of *Hieracium pilosella*; the rust fungus *Puccinia hieracii* var. *piloselloidarum*, and the powdery mildew *Erysiphe cichoracearum*). Biotic interactions, particularly with parasites, and to a lesser extent predators, have long been cited as a possible explanation for the prevalence of sexual reproduction (Levin 1975). Investigating this as a factor on the expression of apomixis in *H. pilosella* may indicate whether this has played a role in determining the reproductive mode in this species.

The Red Queen hypothesis is one of the most frequently cited explanations for the maintenance of sexual reproduction (see Levin 1975, Bell 1982, 1987, Templeton 1982, May & Anderson 1983, Anderson & May 1986, Lively 1992, 1996, Ridely 1993, Lively & Howard 1994, Howard & Lively 1995, Dunbrack *et al.* 1995, Peck & Waxman 2000). As has already been discussed in chapter I.4, sexual reproduction is a comparatively expensive reproductive strategy, and many theories have been formulated to explain its' prevalence. The Red Queen hypothesis, first suggested in a slightly different context by van Valen (1973), relies on frequency dependent selection to provide a fitness advantage to sexual types. The ubiquitous nature of parasites was thought to provide a constant race between predators, parasites, and their target species (van Valen 1973). Pests and pathogens were postulated to have their effectiveness decreased if the host they attacked had an 'open' recombination system, as the host would produce variable offspring, some of which may be resistant to the parasite. The parasite in turn would be selected to infect the hosts that are present at the highest frequency, driving constant shifts in genotype frequencies of both host and parasite populations. An analogy was drawn with the tale of the Red Queen in Lewis Carroll's (1898) "Through the Looking Glass" who although constantly running, never gained ground, just as the host could keep responding without escaping parasitism due to adaptation in the parasite. Levin (1975) recognised that sexual reproduction would decrease the frequency of host genotypes compared to asexual reproduction, thus providing a benefit to sex if parasites were selected to infect common genotypes (frequency dependent selection). Evolutionary theory predicts that all parasites will be under selection to attack the most common genotype, as this will

maximise fitness (Ridley 1993). As parasites generally have shorter generation times than their hosts, it is suggested that sex is necessary to generate the variation required to avoid parasitism (Bell 1982, Ridley 1993).

May and Anderson (1983) modelled the relative effective selection that would be required for sexual reproduction to be favoured in the presence of parasites. The model used differs from many others in that it includes both frequency and density dependence for parasite transmission and virulence. Density dependence was modelled to increase the strength of negative interactions, and therefore facilitate the two-fold advantage necessary for sexual populations (see for example Lively 1992, 1996; also Chapter I.4). It was demonstrated in both one and two loci systems that the effect on fitness of parasites had to approach lethality for the two-fold disadvantage of sex to be overcome. If the inheritance of sex was a multi-locus system, then the expected range of values for virulence and transmission would have greater ranges, although the lethality still had to approach 100 % for sexual individuals to be able to persist in the population. May and Anderson (1983), and later work by the same authors (Anderson & May 1986), point out that intermediate virulence is usually selected for in parasites, making this an unlikely single explanation for the prevalence of sex.

More recent works have revised the effect required by parasites to explain the maintenance of sex. Howard & Lively (1994) and Lively & Howard (1995) conclude that moderate effects of parasites can still maintain sex in populations, provided that a moderate mutation rate is present. Mutation rate has also been postulated as an explanation for the maintenance of sex, with asexual taxa accumulating mutations through time, and subsequently suffering from lower fitness. Sexual taxa can "purge" mutations through recombination events, with deleterious mutations being removed through the mortality of individuals with high mutational load (Kondrashov 1982, 1988, Chao 1990). A combination of the effect of parasites and a relatively low mutation rate showed that sexual reproduction would be maintained over a wide range of recombination rates and parasite virulence. The combination of parasites and mutation is thought to provide enough benefit to maintain sex in conjunction with frequency dependent selection. If frequency dependent selection, or some other mechanism that

drives the population through oscillations is not operating, then this model becomes invalid (Howard & Lively 1994, Lively & Howard 1995).

The parasite used in this case is *Erysiphe cichoracearum*, a powdery mildew commonly found on populations of *Hieracium pilosella* throughout the South Island (Jenkins 1995). This parasite grows as mycelium on the surface of the plant, with only haustoria penetrating the surface to derive nutrient. The impact it has on plant vigour can be moderate to high, with some lethality reported under field conditions (Jenkins 1995). Although it is rare for *Erysiphe* spp. to have lethal effects on *H. pilosella* (although see Jenkins 1995), this work attempts to quantify relatively small modifications to the reproductive strategy (i.e. a low rate of outcrossing), and consequently relatively small fitness costs due to recombination.

Nutritional factors

Nutrient level has been extensively studied as a factor influencing the invasive ability of *Hieracium pilosella*. For example, the change in nutrient levels in many areas of the high country during the 1980s due to the removal of government fertiliser subsidies has been suggested as a reason for the range expansion of *H. pilosella* (see Scott *et al.* 1990, Jenkins 1992, Boswell & Espie 1998; although see Fan & Harris 1996). This is often a critical factor in the management of crop production, and is a factor that can be readily changed by management practices. Additionally, there is some evidence that elevated levels of inorganic salts can influence the levels of apomixis in some species, most likely from inducing water stress (Gounaris *et al.* 1994). That such a mechanism has found to exist may indicate that factors affecting plant metabolism directly, such as fertiliser levels, may be important. While this study will not provide resolution to individual nutrients, it will give an indication of the relative importance of this compared to other factors, such as genotype, for determining the levels of residual sexual reproduction in *H. pilosella*.

A transgenic pollen donor and biochemically selectable phenotype screening system as described in chapter IV.2 was also used in this experiment. The advantages of this system are described in chapter IV.2.

V.1.2. MATERIALS AND METHODS

The reproductive performance of *Hieracium pilosella* under uniform conditions: isolating the effect of genotype

Five genotypes of *Hieracium pilosella* were chosen to represent a range of potentials for sexual reproduction as detected in the field study (Chapter II.1). 60 ramets of each genotype were grown under uniform conditions to minimise phenotypic variation within samples. DNA ploidy equivalents of the plants were recorded following the method described in chapter II.3, and substantial between genotype variation detected (see Table 5.1.1). Genet code corresponds to capitulum number in the 1998 / 1999 field season data (see Appendix 2).

Table 5.1.1 Ploidy levels and reproductive investment of genets under field conditions.

Genet code	1998/1999 Code and site	DNA Ploidy equivalents	% of Sexually derived progeny, 98/99
G1	1106-Little river	36.00	9.68%
G2	1040-Cass flats	45.43	8.70%
G3	1005-Cave stream	47.21	0.00%
G4	1024-*Draco* flat top	46.87	2.86%
G5	1060-Chilton valley	46.24	0.00%

The sixty plants of each genotype were transferred to quarantine conditions, and fifteen of each assigned to each of the four treatments described below. Half of the plants from each genotype were potted in a mix with no additional fertiliser. The remaining thirty plants were potted in mix with the addition of the equivalent of 200kg ha^{-1} of di-ammonium phosphate [DAP; Nitrogen 18, Phosphorous 20, Sulphur 2]. Fertiliser element levels per hectare were therefore: N, 36kg ha^{-1}; P, 40 kg ha^{-1}; S, 8 kg ha^{-1}. These nutrient levels were chosen to represent a typical of a pastoral fertiliser treatment (T.A. Jenkins *pers comm.*). One half of each genotype in each nutrient treatment was

inoculated with powdery mildew (*Erysiphe cichoracearum*), giving a total of four treatments per genotype. With the five different genotypes used, the total number of treatment combinations was twenty.

Plants were inoculated with powdery mildew by rubbing the leaves of the plants to be infected with leaves already suffering heavy infestation. Powdery mildew was collected on *Hieracium pilosella* growing in the field in the Tekapo area of the central South Island, and maintained *in planta* under glasshouse conditions.

Selection of pollinator accession and progeny screening

All plants were pollinated with kanamycin resistant tetraploid *Hieracium aurantiacum*, containing a single copy of the NPT II (Neomycin Phosphotransferase II) resistance gene [transformation via *Agrobacterium tumefaciens* strain 722, pTiA6NC plasmid, pGA643 vector] (see Bicknell & Borst 1999). This accession was used as it was found to produce a high degree of viable pollen, in both transformed and wild-type individuals. Also tested was a kanamycin resistant tetraploid *H. caespitosum,* C4 (NPT II gene, *Agrobacterium tumefaciens* strain LBA4404 / pGA643 vector). The C4 accession of *H. caespitosum* was found to produce a low number of viable seed when crossed to the tetraploid sexual from *Dracophyllum* flat. The percentage of progeny resistant to kanamycin also did not approach the 50 % level expected from the nature of the insert. This indicated a problem with the pollen production of this accession, and most likely a high proportion of progeny were produced via mentor effects, indicating that the pollen from C4 was generally of low viability. Details of using the kanamycin marker system for the detection of sexual events can be seen in chapter IV.2.

Plants were grown under high-pressure sodium vapour lamps with a 14 hour photoperiod, in a constant 22° C temperature. Capitula were covered with the same bag arrangement as for the field trials (see Chapter II.1). Seed sterilisation and germination also followed the methods of the field trial, with the addition of kanamycin at 50mg/L to the agar media. This precludes the need to grow plants to the adult stage, as is necessary with the *Hieracium aurantiacum* system (see Chapter IV.2). Seedlings were allowed to develop on the kanamycin media for at least four weeks, and progeny scored as either resistant or susceptible to the antibiotic following the method of chapter IV.2.

Statistical analysis

Plants were arranged in a randomised block design to ensure that the effects of possible between-cell differences did not confound results. Due to only two quarantine cells being available for this experiment, it was necessary to alter powdery mildew treatments over time between the two cells to avoid pseudo-replication. Although this is not the most desirable method, it does avoid a completely segregated design (Hurlbert 1984). Block (i.e. which cell the pollination and seed set occurred in) was included as the first factor in the analyses of seed production and reproductive mode, to remove any variation due to between-cell differences prior to testing for effects of factors. Seed count data were square root transformed prior to analysis using ANOVA. Proportion of progeny estimated to be produced sexually (i.e. the number of resistant progeny multiplied by two / total progeny) was Arcsine transformed prior to analysis following the method of Anscombe (1948) (see Chapter IV.1). In both analyses, genotype, nutrient and presence / absence of the powdery mildew were included as factors.

V.1.3. RESULTS

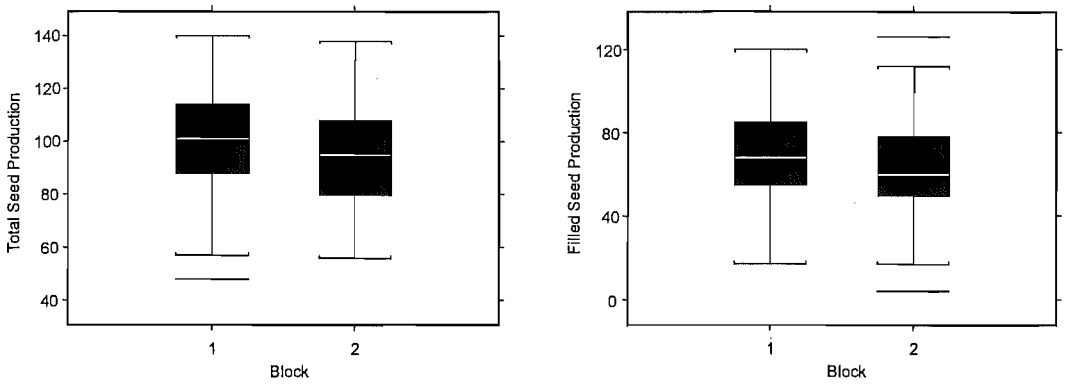
The A3.4 accession with the kanamycin insert proved to be a suitable pollen donor for this study, with both high seed set and the proportion of resistant progeny approaching 50% (63 resistant progeny out of a total of 134 from the three test crosses – 50.747%) when crossed to obligate sexual lines of *Hieracium pilosella.* The amount of viable seed was also much greater than for the C4 accession (an average of 44.7 per cross). The ability of this plant to produce a moderate degree of resistant progeny when crossed with facultative apomictic *H. pilosella* also indicates its' suitability for this study. That only three test crosses were possible precludes the use of statistics to determine the suitability of this accession, however the results of these crosses, particularly the proportion of resistant progeny approaching 50 % when crossed to an obligate sexual, indicated that this was a suitable pollen donor.

Seed production

Seed production displayed considerable variation over the genotypes and treatment regimes. Total seed production was found to vary significantly by genotype ($p = 0.00032$), with genotypes 1 and 3 producing significantly more seed than genotype 5 (see Figure 5.1.2, Table 5.1.3). It was also found that total seed production was significantly lower in the treatments with the added fertiliser ($p = 0.036$) (see Figure 5.1.3, Table 5.1.2).

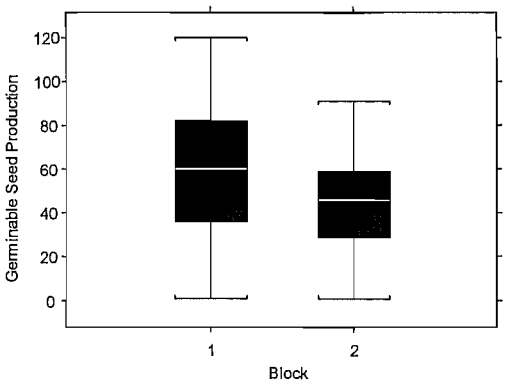
A significant effect of block was found for both filled and germinable seed production ($p = 0.040$ and $p = 0.0028$ respectively) (Table 5.1.2). Both filled and germinable seed production was found to be significantly higher in cell 1 (Figure 5.1.1), indicating that there was a difference in the environment of the two quarantine cells. The block effect was not found to influence total seed production or reproductive mode, however, and no further significant effects of treatment were observed for either filled or germinable seed production.

Figure 5.1.1 Total, Filled and Germinable Seed Production by Block



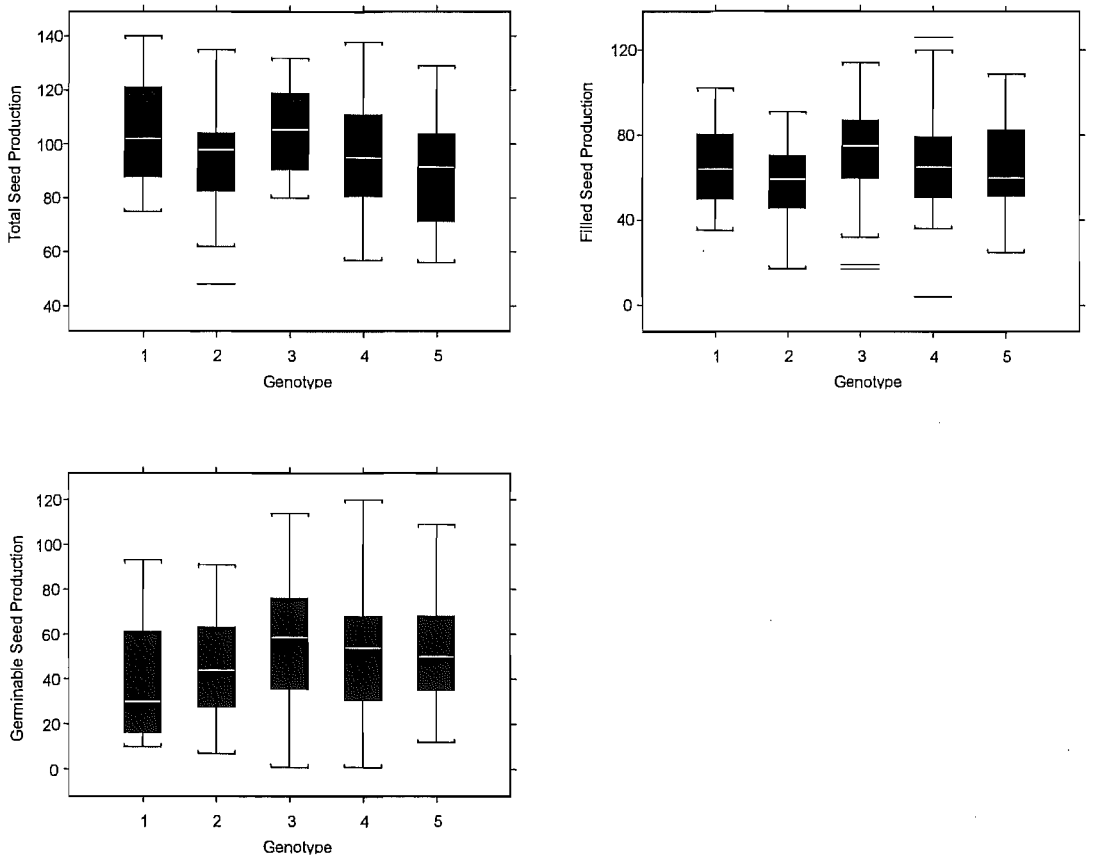
Block 1 (Northern cell), n = 78, Block 2 (Southern cell), n = 103

Figure 5.1.1 cont



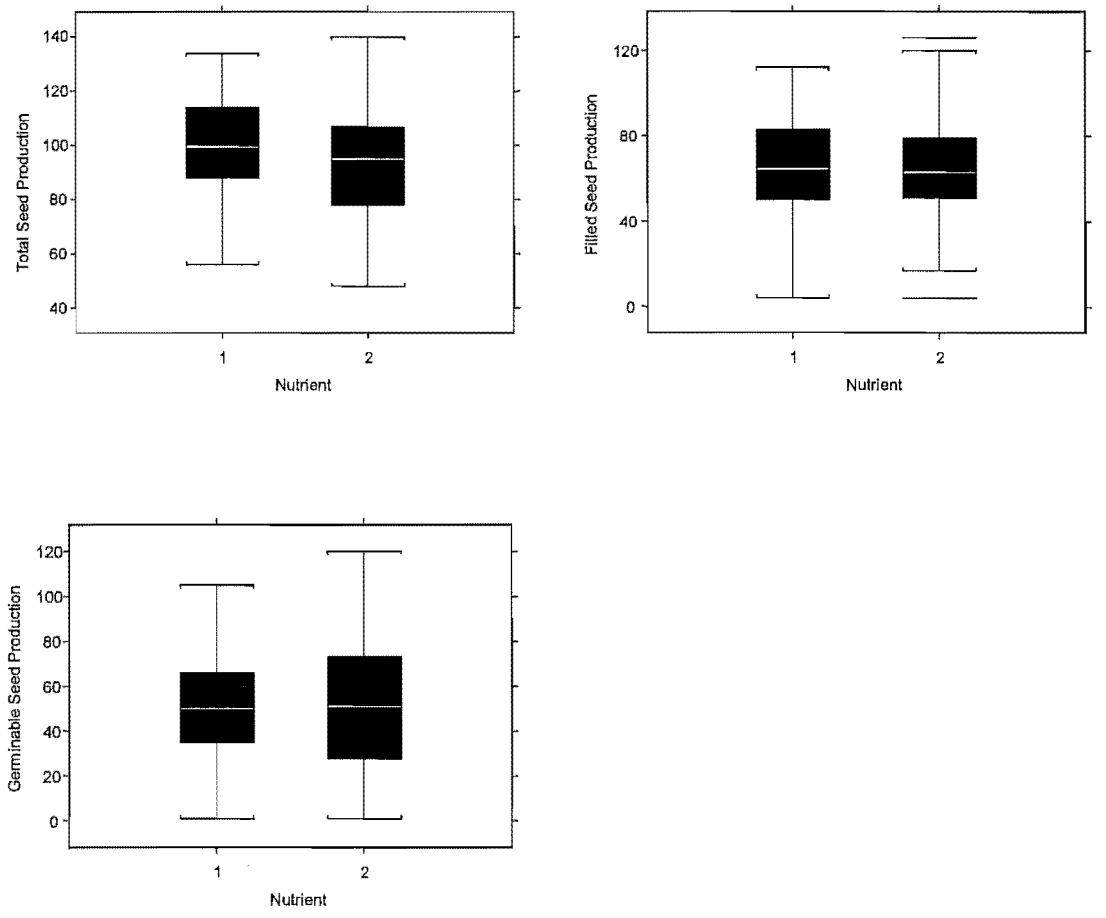
Block 1 (Northern cell), n = 78, Block 2 (Southern cell), n = 103.

Figure 5.1.2 Total, Filled and Germinable Seed Production by Genotype



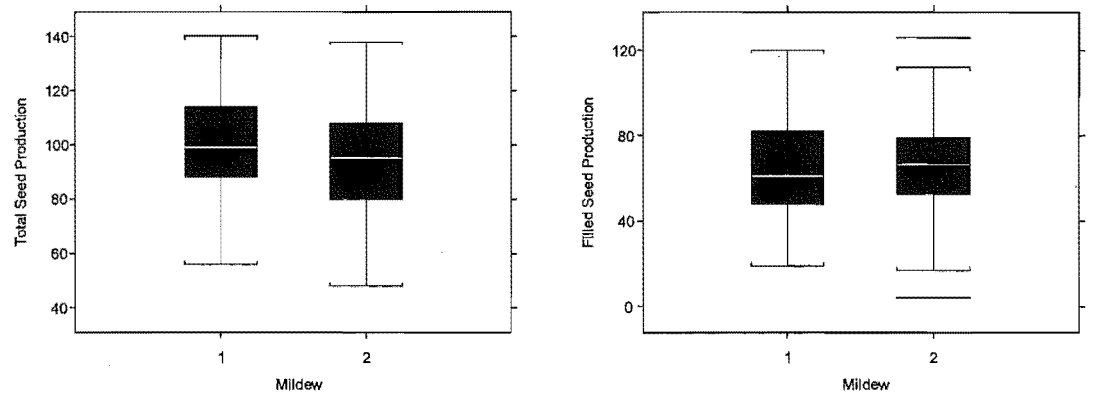
Genotype 1, n = 23; Genotype 2, n = 33; Genotype 3, n = 34; Genotype 4, n = 47, Genotype 5, n = 44.

Figure 5.1.3 Total, Filled and Germinable Seed Production by Nutrient level



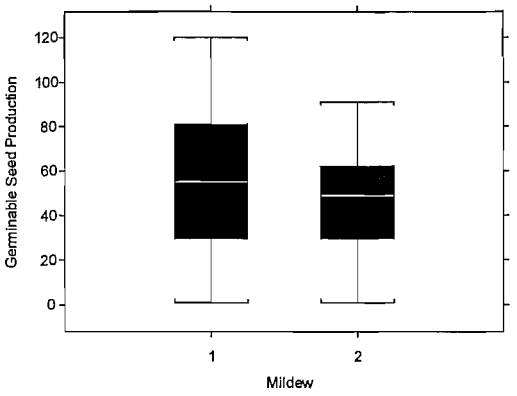
Nutrient 1 (no added fertiliser), n = 94, Nutrient 2 (200kg ha^{-1} DAP), n = 87.

Figure 5.1.4 Total, Filled and Germinable Seed Production by Presence of Powdery Mildew



Mildew 1 (powdery mildew absent), n = 87, Mildew 2 (powdery mildew present), n = 94.

Figure 5.1.4 cont



Mildew 1 (powdery mildew absent), n = 87, Mildew 2 (powdery mildew present), n = 94.

Table 5.1.2 Analysis of Variance Table, Seed production under Glasshouse conditions by Treatment (square root transformed).

Total Seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Block	1	2.0345	2.034548	2.208531	0.14
Genotype	4	20.4033	5.100833	5.537028	**0.0003**
Nutrient	1	4.1017	4.101651	4.452401	**0.04**
Mildew	1	1.3718	1.371765	1.489070	0.22
Residuals	173	159.3714	0.921222		

Filled Seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Block	1	8.8371	8.837143	4.291469	**0.03979**
Genotype	4	8.8880	2.222009	1.079046	0.36846
Nutrient	1	0.1145	0.114474	0.055591	0.81389
Mildew	1	3.5375	3.537485	1.717864	0.19171
Residuals	173	356.2477	2.059235		

Table 5.1.2 cont

Germinable Seed

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Block	1	35.8392	35.83924	9.175962	**0.003**
Genotype	4	18.6152	4.65379	1.191515	0.32
Nutrient	1	0.0158	0.01580	0.004045	0.95
Mildew	1	3.7364	3.73639	0.956633	0.33
Residuals	173	675.6991	3.90577		

Table 5.1.3 Multiple Comparison test, Square Root Total Seed by Genotype. Genotypes that share a line beneath them are not significantly different at $\alpha = 0.05$).

Genotype3	Genotype 1	Genotype 2	Genotype 4	Genotype 5
_____		_____		
		_____		_____

Sexual reproduction

All genotypes produced at least some resistant progeny, although not under every treatment combination (Table 5.1.4). This does demonstrate, however, that some sexual capacity remains in the genotypes chosen, although this was not always detected even following several crosses being performed. The treatment of added nutrient and no powdery mildew produced resistant progeny from four of the five genotypes, although no data for genotype 1 was available for this combination of treatments (Table 5.1.4). No significant effects of block, genotype, nutrient or the presence of powdery mildew could be detected on the proportion of progeny produced sexually from the crosses (Table 5.1.5). The frequency of sexual events detected was comparably low (Table 5.1.4) (cf. Chapters II.1, IV.2, IV.3) and can possibly be attributed to the different efficiency of the transgenic accession to act as a pollen donor, or the environmental conditions under which the plants were grown. Temperature was possibly lower than for the other glasshouse trials due to the design of the quarantine cells, and also most crosses were carried out during the winter, possibly decreasing the frequency of outcrossing (see Chapter IV.1). The amount of replication for each factor can be seen in Table 5.1.6. There was no dependence of the frequency of sexual reproduction as measured under

field conditions on the proportion of progeny that were produced sexually in this study ($R^2 = 0.1201$, p-value = 0.5677).

Crosses resulting in at least one resistant individual had a significantly greater number of progeny than crosses where no resistant progeny were detected, when compared with a t-test (averages of 60.39 and 49.01 respectively, t = 2.1704, df = 183, p-value = 0.031).

Table 5.1.4 Frequency of Resistant Progeny by Genotype and Treatment, and estimated Percentage (resist*2 / total progeny).

Treatment	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5	Treatment totals
Nutrient + Mildew +	3 / 474 = 1.266	4 / 312 = 2.564	2 / 321 = 1.246	0 / 511 = 0.000	4 / 883 = 0.906	**13 / 2501 = 1.040**
Nutrient + Mildew -	NA NA	3 / 571 = 1.050	5 / 336 = 2.976	1 / 775 = 0.2.58	1 / 251 = 0.796	**10 / 1933 = 1.034**
Nutrient – Mildew +	0 / 233 = 0.000	6 / 627 = 1.914	2 / 418 = 0.956	2 / 542 = 0.738	1 / 529 = 0.378	**11 / 2349 = 0.936**
Nutrient – Mildew -	0 / 274 = 0.000	0 / 9 = 0.000	1 / 826 = 0.242	2 / 609 = 0.656	1 / 699 = 0.286	**4 / 2417 = 0.330**
Genotype totals	**3 / 981 = 0.612**	**13 / 1519 = 1.712**	**10 / 1901 = 1.052**	**5 / 2437 = 0.410**	**7 / 2362 = 0.592**	**38 / 9200 = 0.826**

Table 5.1.5 Analysis of Variance, Reproductive mode by Treatments

Analysis of Variance Table

Response: Arcsine transformation of proportional data (Anscombe 1948).

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
block	1	0.00500	0.005004	0.73988	0.39
genotype	4	0.04537	0.011343	1.67718	0.16
nutrient	1	0.00794	0.007940	1.17403	0.28
mildew	1	0.00131	0.001310	0.19367	0.66
genotype:nutrient	4	0.02955	0.007386	1.09215	0.36
genotype:mildew	4	0.00758	0.001895	0.28015	0.89
nutrient:mildew	1	0.00015	0.000150	0.02223	0.88
genotype:nutrient:milde	3	0.00761	0.002536	0.37497	0.77
Residuals	161	1.08883	0.006763		

Table 5.1.6 Number of crosses per Treatment, and Genotype by Treatment

Treatment	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5	**Treatment totals**
Nutrient + Mildew +	0	16	6	16	4	**42**
Nutrient + Mildew -	10	6	5	8	16	**45**
Nutrient – Mildew +	7	1	17	12	15	**52**
Nutrient – Mildew -	6	10	6	11	9	**42**
Genotype totals	**23**	**33**	**34**	**47**	**44**	**181**

V.1.4 DISCUSSION

Sexual reproduction

Despite the large sample sizes in this study (181 crosses, 9200 progeny), no effect of plant genotype, nutrient level or the presence of the powdery mildew was observed on the reproductive mode of this species. Although the plants were chosen to represent a range of sexual frequencies from the field study, it is clear that the potential observed in the field did not directly transfer to the frequencies observed under glasshouse conditions. This indicates that a single cross under field conditions (see Chapter II.1) is not sufficient to determine the amount of facultative sex in a genotype. It is possible that the environment contributed significantly to the reproductive mode as observed under field conditions, giving an unreliable measure of the average frequency of sex of the genotypes selected. Paternal function, particularly the growth and germination of pollen grains on the stigma may be strongly influenced under field conditions, contributing to the variation observed; a factor not investigated directly in this study. Although an earlier chapter (IV.1) examining the effect of the site on reproductive mode found a significant effect, but this study failed to determine any influence of genotype is an interesting finding. This further reinforces the importance of the environment and population level variation in determining the reproductive mode of *Hieracium pilosella*.

The lack of a nutrient effect on reproductive mode but a decrease in seed set in the high nutrient treatment indicates that increasing nutrient level may increase investment in vegetative growth, rather than reproduction via seed. If an increase in nutrient shifts investment from reproduction via seed to clonal growth this may be best explained as the fastest way to respond to an available resource, and further demonstrates the plastic nature of *Hieracium pilosella*. Fan and Harris (1996) showed that *H. pilosella* and *H. praealtum* responded faster to increasing nutrients, measured by increasing biomass, than the two other species in the comparison (*Rumex acetosella* and *Festuca novae-zealandiae*). The ability to shift investment from seed to vegetative reproduction rapidly contributes to the plastic strategy of this species, and this is potentially more important for short-term survival than a shift in reproductive mode. However, it is interesting to note that only total seed production, not filled or germinable seed, was found to be significantly different under the different nutrient regimes.

The presence of powdery mildew did not significantly effect the reproductive mode of the *Hieracium pilosella* genotypes selected for this study. This demonstrates that the presence of this pathogen is not an important determinate of the reproductive mode of this species. If powdery mildew had a significant enough effect on the plants' development to the point where it significantly decreased fecundity (no effect on fecundity was detected) then it is possible that there could be some influence on reproductive mode due to the association found between reproductive mode and fecundity. It is unlikely that the effect of powdery mildew under field conditions would be greater than observed in the glasshouse, as these conditions appear to be more optimal for powdery mildew growth and reproduction (see also Jenkins 1995).

That an association was found between fecundity and reproductive mode, with a significantly greater number of progeny produced in crosses where at least one resistant individual was detected (see also Chapter II.1), demonstrates that there is perhaps a trade-off between fecundity and reproductive mode. The cost of sex is offset under these conditions by the increase in reproductive output. That the increase in fecundity is much greater than simply an increase by the amount of sexually produced (resistant x 2) progeny demonstrates a shift in investment type with conditions favouring higher reproductive output. Although the conditions for the treatments in this study should be

theoretically uniform excluding the manipulated factors, several other parameters may have influenced plant development. Variation in the watering regime within the cells, differences in physiological conditions of the ramets prior to splitting into individual pots, variation in lighting within the cells and numerous other factors may have influenced plant growth. Between-cell variation was accounted for by the "block" effect, although there was still considerable unexplained variation as is nearly always the case with factorial experimental designs.

The lack of an influence of nutrient level and powdery mildew, as tested in this study, makes it possible to conclude that these factors do not significantly effect the reproductive mode of *Hieracium pilosella.* The rate of fertiliser application used in this study is towards the high end of the range that is commonly applied in pastoral agricultural situations (T.A. Jenkins *pers. comm.*), although experimental treatments are often higher (see Scott *et al.* 1990, Scott 1993b, 2000a, 2000b). Although supplemented nitrogen and phosphorous were high, the fertiliser selected had only moderate amounts of sulphur, an element that has been associated with the suppression of *H. pilosella* in agricultural trials (Scott 2000a, 2000b). However the studies by Scott (2000a, 2000b) were on soils where sulphur is known to be a limiting factor, which is not the case in this study. The lack of an effect of fertiliser application on reproductive mode does show that this is not a critical factor for the breakdown of apomixis, although further testing may be desirable with higher supplementary sulphur. The level of powdery mildew infestation was considerably greater than is commonly observed in the field and the subsequent lack of an effect on reproductive mode demonstrates that this is unimportant in determining reproductive strategy.

The results of this investigation indicate that the addition of fertiliser or the presence of a pathogen will not substantially influence the reproductive mode of *Hieracium pilosella.* The addition of fertilisers in conjunction with over-sowing can suppress *H. pilosella*, especially with moderate rainfall levels (Scott 1993b). That the rate of sexual reproduction does not increase following the addition of fertiliser, or in the presence of a generalist pathogen, indicates both methods may be suitable control strategies without selecting in the short term for more reproductively plastic genotypes.

Chapter VI. GENERAL DISCUSSION.

VI.1.1 THE REPRODUCTIVE PATTERNS OF *HIERACIUM PILOSELLA* IN NEW ZEALAND

There is both maternal and paternal potential for sexual reproduction in field populations of *Hieracium pilosella* in New Zealand. On average, 3.81 % (370 / 9705) [facultative apomicts only: 2.12 %; 201 / 9489] of viable progeny following artificial pollination under field conditions were the result of sexual reproduction, although there was considerable variation in the among-population frequencies. The use of a morphologically distinct species (*H. aurantiacum*) as a pollen donor was shown to be an efficient method for the detection of sexual events in studies with large sample sizes. This has certainly proved more time efficient than molecular methods, although RAPDs were found to provide a relatively quick and easy method for confirming hybrid origin. Whether there is a difference in pollination efficiency using a different species, and therefore a difference between the rate of sex measured and the actual field rate, is unknown; however this method does allow for a comparison of the frequencies of sexual reproduction among sites. All populations examined possessed at least some female potential for sexual reproduction, indicating that facultative apomixis is widespread in New Zealand populations of *H. pilosella.* Both tetraploid and pentaploid apomicts were shown to produce progeny sexually, contrary to the findings of Gadella (1972, 1987, 1991a, 1991b) for *H. pilosella* in Europe, who reported tetraploid "apo-amphimicts" and almost always, "obligate" apomictic pentaploids (although see IV.1.5 for further detail; and it should be noted that obligate sexual pentaploids have been discovered in the Czech Republic [F. Krahulec & A. Krahulcová *pers. comm*]). Hybrid offspring were often obligate sexuals, indicating the potential for the segregation of this trait in field populations. It is therefore of no surprise that at two of the seven sites examined obligate sexual tetraploid *H. pilosella* was discovered. This is the first record of this type in New Zealand, and it is most probable that it has arisen on site via residual sexual reproduction in facultative apomicts (see Chapman *et al. in prep*).

The reproductive patterns of *Hieracium pilosella* have been shown to be complex, and variable, at the population level in the field. The levels of sexual reproduction observed

are higher than those reported under glasshouse conditions (see Chapman & Bicknell 2000), indicating that previous studies of this group may have underestimated the potential for recombination.

Male function was determined using the fluorochromatic reaction test. While this is not a perfect test of pollen function, it does indicate both the membrane and enzymatic integrity of the male gamete. It is interesting to note that no difference was found in the percentage of stainable pollen produced by plants from three of the four sites comprised of facultative apomictic individuals, and the obligate sexuals. As is observed in the majority of apomictic taxa (Asker & Jerling 1992), *Hieracium pilosella* maintains the male function despite the predominantly asexual reproductive mode.

Hybrid progeny were often of BIII ("addition hybrid") origin, as detected by flow cytometry. Due to the absence of BIII hybrids in the progeny of crosses to obligate sexual tetraploids (as the maternal parent), it would seem irregularities in meiosis are much more common in the egg cell line of facultative apomicts. These irregularities can lead to the observation of aneuploids in some field populations (see Chapman & Lambie 2000 as *Pilosella officinarum*). No BIII hybrids were observed with A3.4 as the paternal parent in crosses with obligate sexuals, indicating the regular male meiosis in this aneuploid accession.

The inheritance of apomixis observed in this study supports the finding of Bicknell *et al.* (2000). Apomixis is inherited as a single, dominant, allele (see IV.1.6). The frequency of obligate sexual progeny among the total hybrid progeny gives an indication of the level of heterozygosity for apomixis in field populations of *H. pilosella*. If obligate sexual plants represent approximately 20 % of the total progeny when crossed to Aaa (the genotype of A3.4 [Bicknell *et al.* 2000]), as was found, it is possible to predict that on average a pentaploid plant would most likely be Aaaaa for apomixis. If AAaaa or more dominant-laden genotypes were common, it would be unlikely that 20 % of the outcrossed progeny would be obligate sexuals.

VI.1.2 COMPARISONS TO THE PATTERNS OF GENETIC VARIATION IN NEW ZEALAND

It is clear from the findings of this study that the complex population structure observed in field populations of *Hieracium pilosella* is most parsimoniously explained by low levels of sexual reproduction in predominantly aposporous populations. The relationship between population variation and the level of residual sexual reproduction at a site is further evidence of this. Not only does this indicate that sexual reproduction occurs under field conditions, but seed progeny, at least sexually produced seed progeny, are recruited into the population. Further evidence was found from compatibility analysis of the banding patterns that found that all populations had incompatibilities that could not be parsimoniously explained by mutation. Although it is possible that mutation also plays a role in the variation recorded in populations, compatibility analysis detects patterns that most likely did not arise via mutation, and predicts that these are the result of recombination. The findings of this study, however, are perhaps indicative of both mechanisms playing a role. Recombination, however, has greater potential for the generation of variation, and is possibly more important in the evolutionary potential of these populations.

The conclusion that sex has contributed substantially to populations of *Hieracium pilosella* in New Zealand is in contrast to demographic studies that concluded that seed production was unimportant in the spread and maintenance of populations (Lamoureaux 1998, Makepeace 1980). Other work has concluded that seed does contribute to populations (Rose & Frampton 1999) and this may illustrate that recruitment may vary over spatial or temporal scales, explaining the discrepancies in conclusions. Even if a correlation between population level variation and the frequency of sexual reproduction did not exist, the fact that the potential for sex has been identified makes this a more likely explanation than mutation. As mentioned earlier, while mutation has been widely cited as a possible explanation (see Ellstrand & Roose 1987, Menken & Morita 1989, Asker & Jerling 1992, Menken *et al.* 1995), little more than anecdotal evidence exists for this as a mechanism in *Hieracium* spp. The identification of different genotypes at all of the sites also makes the possibility of high founder variation being responsible for the patterns observed unlikely. If high founder variation were the case, we would still expect

to find some widespread clones, or perhaps the occurrence of the same clones in similar habitats, a pattern that has not been observed in this species (Chapman & Brown 2001).

VI.1.3 THE REPRODUCTIVE STRATEGY OF *HIERACIUM PILOSELLA* IN NEW ZEALAND

Field populations of *Hieracium pilosella* in New Zealand appear to possess considerable potential for evolution via facultative sexuality in predominantly apomictic individuals, as is suggested by the levels of population differentiation observed in this and other studies (Chapman *et al.* 2000 as *Pilosella officinarum*, Chapman & Brown 2001). The findings of this work show not only that *H. pilosella* possess potential for the generation of variation, but that this is coupled to the environment on a relatively short temporal scale (the seven days prior to capitulum anthesis). The ultimate mechanism for this is unclear, although the influence of the significant environmental parameters on ovule physiology is perhaps the most parsimonious explanation.

Although the environment plays a role in the expression of sex in facultative apomictic *Hieracium pilosella,* a larger component of variation in the studies presented herein could be attributed to genotype, at least at the population level. Although apomixis does seem to be inherited simply, as a single dominant allele (Bicknell *et al.* 2000), modifiers to the primary apomixis locus in *H. pilosella* have been postulated, and through these the different levels of residual sexuality would be generated (Koltunow 2000, Koltunow *et al.* 2000, Bicknell *et al.* 2001). Although evidence for modifiers has been primarily identified in cultivated accessions, it is clear from this study that considerable variation in these may be present in field populations. The presence of such variation in the potential for sexual reproduction indicates that it is possible that the reproductive patterns observed in *H. pilosella* could potentially represent an evolutionary optimal strategy (Howard & Lively 1994, Lively & Howard 1994, Green & Noakes 1995). Unlike other studies where this has been suggested, there is evidence in *H. pilosella* that there is variation in reproductive mode for selection to act upon.

In the field and glasshouse experiments carried out during this study, it appears that sexual reproduction is most prevalent in conditions that favour high growth rates, and that crosses that produce at least one hybrid progeny have significantly more total progeny than those that do not. Theoretically, the cost of sex is reduced under these conditions due to the general increase in fecundity (see Chapters II.1, IV.3).

VI.1.4 THE ROLE OF THE ENVIRONMENT IN THE EXPRESSION OF APOMIXIS IN *HIERACIUM PILOSELLA*

The environmental control of apomixis in *Hieracium pilosella* is a significant factor in the expression of this trait. Site effects, most likely due to genotype as shown in chapter IV.1, were often the most significant determinant of levels of residual sexual reproduction. However, clear, positive, relationships were found to exist between the levels of residual sexual reproduction, and temperature and rainfall at the three field sites adjacent to weather stations (Chapter IV.1). There was an indication of a negative relationship with altitude, but further investigation would be necessary to determine if this was the case.

Temperature has been identified as being an important factor in determining the reproductive patterns of many species. Mikesell (1997) found that low temperature decreased the reproductive effort of *Pharbitis nil* (Japanese morning glory) dramatically. At 15° C all plants were found to be male sterile, and although flowers were produced, no fruit or seed developed. Vegetative growth was also suppressed. Mikesell (1997) suggested that the decreased mobilisation of stored reserves in the plant may have been responsible for these patterns, as this is more often the food source for embryonic structures. Dorken and Eckert (2001) suggested that temperature may be responsible for the decrease in sexual reproduction and subsequent low population genetic variability in the aquatic *Decodon verticillatus* (Lythraceae) at higher latitudes. This was identified as the most likely explanation as no other environmental parameters in their study area were thought to be correlated with latitude.

Glasshouse investigations into the reproductive mode of *Hieracium pilosella* showed no significant effects of nutrient state of the plants or the presence of powdery mildew on reproductive mode. The five genotypes selected for this study, based on the frequency of sexual reproduction recorded in the field study, also showed no significant differences from each other in the four treatment combinations. No effect of photoperiod was found on the expression of sexual reproduction on plants from the Chilton valley site when grown under 14 and 16 hour day lengths.

The finding that both temperature and rainfall were important for the frequency of sexual reproduction but photoperiod was unrelated is interesting in regard to the conclusions of other studies. The importance of photoperiod in determining the reproductive mode of facultative apomicts has been widely cited (see Asker & Jerling 1992, Mogie 1992). The citation classic amongst these is usually Knox (1967), looking at the influence of the environment on apomixis in *Dichanthium aristatum* (Gramineae) under field conditions. Knox concludes that photoperiod is the most important cue for apomixis, but also states that this conclusion is confounded by other factors. There was a positive relationship between temperature and also rainfall, but due to photoperiod being more predictable, Knox declared that this was more likely to be the cue for reproductive mode. Knox and Heslop-Harrison (1963) also showed that photoperiod was important for determining the reproductive mode of *D. aristatum* but under controlled experimental conditions.

Other studies that have examined the effect of photoperiod on apomixis have been inconclusive. Burton (1982), examining apomixis in bahiagrass (*Paspalum notatum*) found no effect of photoperiod, water stress or low fertility soil, on the reproductive mode of this species. A study looking at the facultative apomitic grass species *Hyparrhenia hirta,* found no significant influence of photoperiod, although they did try to claim an effect for one of the strains of examined, despite very small sample sizes (McWilliam *et al.* 1970). The three other strains tested in this study displayed no response to any light treatments, indicating that this is far from being a critical cue in this species. Evans and Knox (1969) examined the influence of photoperiod on reproductive patterns in *Themeda australis.* This also failed to show any clear relationship between the expression of apomixis and photoperiod at flowering, although there was some

evidence that the pre treatments before floral induction had a role. Although this may be of interest physiologically, it has few applications to understanding the differential expression of apomixis under field conditions, and is a more complex finding than Knox (1967), who suggested a relatively simple cue for this trait. Hussey *et al.* (1991) also found no evidence of photoperiod influencing reproduction in buffelgrass (*Pennisetum ciliare* [= *Cenchrus ciliaris*]). Although Gounaris *et al.* (1991) claimed the presence of inorganic salts influenced the embryology of both apomictic and sexual buffelgrass, this paper fails to include any statistical analysis of this phenomenon, making it difficult to gauge the importance of this as a factor. In any case, the influence of inorganic salts may be of interest experimentally, but is not of use in commercial situations due to adverse affects on growth, nor is it of relevance in a bio-control situation, where the target species is not found on such a gradient.

That this work has identified the importance of temperature and rainfall on the expression of apomixis, but has found the effect of photoperiod inconclusive, can be interpreted in several ways in respect to the existing literature. It could be argued that the findings of Knox (1967) are better interpreted as showing a relationship with temperature and rainfall. Another reason for reaching this conclusion could be the lack of an effect found by those investigating the effect of photoperiod under glasshouse conditions (see Evans & Knox 1970, Burton 1982, Hussey *et al.* 1991). Conversely, it would be possible to suggest that the Gramineae possess a different mechanism for the physiological control of this trait, due to differences in physiology between the two classes (monocotyledons compared to dicotyledons). It is known that physiological differences exist between these groups, despite the fact all of the species mentioned are classified in the broad sense as "aposporous apomicts".

The geographic relationships discussed so often in the literature in respect to apomictic taxa being restricted to higher latitudes and altitudes are potentially misleading in that these are most likely proxies for rainfall and temperature gradients. The amount of rainfall at different altitudes and latitudes does vary in different parts of the world, but a more general, negative, relationship can be seen with temperature. The distinct restriction of apomicts is not to high altitude / latitude areas *per se,* but to the environmental conditions that are found in these areas. The ability of temperature to

describe the patterns of residual sexual reproduction in *Hieracium pilosella* should not be overlooked. The analysis in chapter IV.1, removing the site effect before considering environmental variables, shows that even after removing the potential genetic bias of this experiment due to site differences, there is still a highly significant positive relationship between the two.

The role of genotype in chapter IV concentrated on its potential to influence reproductive pattern at the population level. This does not account for variation in this trait at the individual level, which was not found to be a conclusive factor for the five genotypes tested at the individual level (see Chapter V). Variation at the population level in the expression of sexual reproduction, however, represents the potential outcome for selection of this trait at any site. In both chapters IV.1 and IV.3 there was a strong genotype effect at the population level for the expression of residual sexual reproduction. This would indicate that there has been differential evolution of, perhaps as the result of differential selection for, this trait at the sites examined.

The results of this study show that in New Zealand populations of *Hieracium pilosella,* temperature and rainfall during the time of egg cell formation is the most important environmental cue for the breakdown of apomixis.

VI.1.5 PLOIDY LEVEL AND APOMIXIS

Despite the potential for the generation of different ploidy levels in New Zealand populations (Houliston & Chapman 2001), populations are predominantly pentaploid. Gadella (1987) states that tetraploids are almost absent from Northern Europe, and that mostly pentaploid, apomictic, bio-types are found; the exception being a few tetraploid "apo-amphimicts". Although the origin of *H. pilosella* in New Zealand is unknown, it has been speculated that Sweden is the most likely source due to the large exports of grass seed from this country to New Zealand in the mid to late 1800s (D. Scott *pers comm.*). If New Zealand *H. pilosella* was sourced from Northern Europe this would partially explain the dominance of the pentaploids in this country.

It is clear from New Zealand studies that ploidy level does not correlate strongly with the mode of reproduction (Makepeace 1985a, 1985b, Jenkins 1992, Bicknell 1997). Although the data presented here suggests that tetraploid and pentaploid apomicts produce approximately the same proportion of sexual offspring, sample sizes are insufficient to rigorously test this idea.

The majority of populations of *Hieracium pilosella* included in this study were comprised of apomicts, although two populations also contained obligate sexuals. At four of the sites at least one apomictic tetraploid was recorded. This appears to be at a much higher frequency than populations in Europe, as reported by Gadella (1987). Although Gadella states that tetraploid apomicts existed, these had a very limited geographic distribution. Gadella (1987, 1991a, 19991b) also stated that pentaploids were almost always obligate apomicts, although some, with a limited geographic distribution, were found to be "amphi-apomicts". Although Gadella (1987) used different methods to determine the reproductive mode of the plants than this study, and there is potential for variation between the two measurements, this suggests that the patterns of variation in the two regions (Central Europe and New Zealand) are very different.

The occurrence of tetraploids in New Zealand populations may be indicative of recombination events between facultative apomicts (see Chapman *et al. in prep*), and is also possibly a quick screening method, via flow cytometry, for the occurrence of obligate sexuals. As in Europe, pentaploid obligate sexuals are absent (although a single population containing pentaploid obligate sexual individuals has been discovered in the Czech Republic [F. Krahulec & A. Krahulcová *pers. comm.*]). This does indicate that ploidy plays an important role in the reproductive mode of this species (see also Gadella 1972, 1987, 1991a, 1991b).

VI.1.6 THE INHERITANCE OF APOMIXIS

The inheritance of apomixis in *Hieracium* subgen. *Pilosella* is as a single dominant allele (Gadella 1991a, 1991b, Bicknell 1997, Bicknell *et al.* 2000). Bicknell and Borst (1996) demonstrated that apomixis would segregate when reduced ploidy levels from a pentaploid plant were created via anther culture. It was found that of the 23 tetraploids produced, (46% of the total sample), all had segregated in respect to the inheritance of apomixis. The findings of this work also support this conclusion, as evidenced by the 1:1 ratio of progeny following the pollination of obligate sexual tetraploids with A3.4, which is known to be Aaa for apomixis (Bicknell & Borst 1996, Bicknell *et al.* 2000). Koltunow *et al.* (1995) states that it is possible to explain all of the known pathways of apomixis in respect to a single, dominant allele. They also state that alleles for sex and apomixis are not mutually exclusive, and this is the pattern we see in facultative apomicts.

More recent work has identified evidence for modifiers to the primary apomixis locus (Koltunow *et al.* 2000, Bicknell *et al.* 2001) which may be partially responsible for the differential expression of apomixis in facultative apomicts. While apospory and autonomous embryo and endosperm development are conferred by a single dominant allele (Bicknell *et al.* 2001), the expression and penetrance of these traits can be altered in different genetic backgrounds. A change in genetic background can have considerable effects on the level, and even mechanism, of apomicticly produced seed, and also the frequency of meiotic embryo sac formation (Koltunow *et al.* 2000). Although the possible presence of modifiers to the primary apomixis locus in *Hieracium pilosella* may appear to further complicate the situation in respect to the isolation of this trait, this system is most likely more simple than that observed in other apomictic taxa (see van Dijk *et al.* 1999).

The absence of apomixis in naturally occurring diploid individuals of *Hieracium* subgen. *Pilosella* from Europe would at first indicate that this subgenus conforms to Nogler's theory (Nogler 1984). This theory states that apomixis is conferred by a single, dominant allele, but that this allele is lethal in a haploid gamete and therefore it is not possible to produce an apomictic diploid via hybridisation. Bicknell *et al.* (2000),

however, found that apomixis could be transferred by both haploid and diploid gametes, indicating that selection acts against diploid apomicts rather than haploid gametes. Bicknell (1997) found it was possible to create diploid apomicts via anther culture of *H. aurantiacum* (A3.4), but also observed that these plants had reduced vigour, stature, seed set, and pollen production. Although the same mechanism of haploid gamete lethality for apomixis has also been observed in several other apomictic taxa [*Ranunculus auricomus* – Nogler (1984), *Panicum maximum* - Savidan (1980), *Parthenium argentatum* – Hashemi *et al.* (1989)], this does not appear to apply to *Hieracium* subgen. *Pilosella.*

Debate, however, on the inheritance of apomixis in aposporous apomicts is by no means closed, and there are many avenues yet to be examined (Mogie 1992). There are contrasting views on whether apomixis in *Taraxacum* is controlled by a single locus; the suggestion by Mogie (1992) being refuted by van Dijk *et al.* (1999). There is mounting evidence, however, that a single locus control is common in aposporous apomicts (Tas & van Dijk 1999).

VI.1.7 THE GEOGRAPHIC DISTRIBUTION OF APOMIXIS

The differences in the range of apomicts and sexual individuals of the same taxa has been termed "geographic parthenogenesis" (Mogie 1992, Asker & Jerling 1992). The examples given earlier (Morita 1976, Gadella 1972, 1982, 1987, Michaels & Bazzaz 1989, Bierzychudek 1989, Mogie 1992, Asker & Jerling 1992) are where there are clear differences between the ranges of sexual and apomictic individuals of a taxon. One of the most convincing theories to explain this pattern, the 'general purpose genotype' as discussed by Bierzychudek (1989), can also be ascribed to several other of these facultative taxa. Other studies that have looked at distributions of apomictic versus sexual individuals often can not clearly state any advantage in environmental tolerance to the apomict, but often only that the apomict is present over a wider geographic range (Bierzychudek 1987b). In contrast, it has also been shown that in a large number of cases, the latitudinal range of apomictic members of a taxon is greater than that of the sexuals, and is also usually further North (Bierzychudek 1987b) (see Table 6.1.1.).

Table 6.1.1 Environmental variation in apomicts: Geographic and Latitudinal Ranges.

Taxonomic Group [Families]	Number of taxa examined	Apomictic range greater?	Apomictic Found Further North? *
Asteraceae	36	30 (83%)	30 (83%)
Gramineae	6	3 (50%)	3 (50%)

*Note that this Table is sourced from a publication in the Northern Hemisphere. The Asteraceae taxa examined did not necessarily have consistency in geographic range and latitudinal range. Taxa of the Gramineae did have complete concordance. From: Bierzychudek (1987b).

The investigation in this study into a relationship between altitude and the frequency of residual sexual reproduction was inconclusive. There was some indication for a negative relationship with altitude, although this was not statistically significant. From the literature, it would seem that the effect of altitude or distribution *per se* is of little importance in controlling the expression of apomixis, and is simply a useful proxy for the environmental parameters that have been shown to influence the expression of this trait (Mogie 1992, Asker & Jerling 1992). It is still unclear what the ultimate cause of the difference in distribution of sexual and apomictic individuals as observed in many taxa is, although the findings of this work are consistent with this being due to differences in environmental tolerance.

Peck *et al.* (1998) has suggested that length of growing season is the critical factor in explaining geographic parthenogenesis. Although this study was not based on empirical data, their model did suggest that asexual individuals were restricted to northern areas due to a short growing season and subsequently a decrease in fecundity in these areas. Sexuals were thought to be unable to invade higher latitudes, as they were adapted to the conditions at lower latitudes, and would suffer a potentially higher "fitness" loss at higher latitudes. The models assume higher fecundity at lower latitudes due to this increase in growing season, and assigned either a fitness advantage or disadvantage to the asexual individuals at random. Whenever the asexuals were assigned a fitness advantage, and even occasionally when given a disadvantage, only asexuals were found at the end of the run (25.5 % of the total number of trials). For sexuals to persist in the remaining 74.5 % of the trials, they had to have a fitness advantage, and even with a fitness advantage, 0.5

% of trials resulted in only asexuals being present. It would therefore appear that for the conclusions of Peck *et al.* (1998) model to be correct, asexuals would almost surely have to have a fitness disadvantage, something that is not predicted by the "cost of meiosis". Peck *et al.* (1998) justify this by arguing that asexuals often suffer from problems of increased mortality due to deleterious mutations or are more susceptible to parasites. It is also interesting to note that no distinction is made between "fitness" and "fecundity" in this work, ignoring the cost of meiosis / cost of males. It is then concluded that as asexuals have decreased fitness, but under the shorter growing season in the North if an optimal phenotype for Northern conditions arises, they may have sufficient advantage over the "maladapted" sexuals to persist in these areas (Peck *et al.* 1998).

It is difficult to ascertain how applicable the model of Peck *et al.* (1998) is due to the assumptions made. The findings of this study indicate that asexuals have a fecundity advantage over sexuals under field conditions (see Chapter II.1; although sexuals can potentially have much greater fecundity in the glass house – see Chapter IV.3), and it would be surprising if the increased efficiency of asexual reproduction would often lead to a decrease in fecundity, let alone fitness, in natural populations. The model of Peck *et al.* (1998) is attractive in that an environmental factor can be invoked to explain the distributions observed. It requires the collection of further data on both the fecundity and fitness differences of sexual and asexual populations to test whether this is applicable. It would also be interesting to include the transmission of an apomixis "gene" in such models to determine how this would effect dynamics.

V.1.8 *HIERACIUM PILOSELLA* AND THE MAINTENANCE OF SEX

A recent review of the theories for the maintenance of sex rather elegantly classified theories as either those based on the "creation and spread of advantageous traits (possibly parasite resistance)" or the purging of deleterious mutations from the genome (Hurst & Peck 1996). These two divisions were made as they represent the two most commonly cited explanations in the literature. As also noted by (Hurst & Peck 1996), other ideas exist, but either lack the advantage necessary to explain sex or are related to relatively

small groups with unusual developmental pathways. This work will concentrate briefly on these two most commonly cited explanations, and how the reproductive patterns of *Hieracium pilosella* can be reconciled with the predictions of these theories.

Although this study is not strictly a test of theories for the maintenance of sex, it is possible to interpret some of the findings in this regard. Firstly, the population structure observed in predominantly asexual populations are complex, and it seems that there is potential for micro-evolution in these populations. That the sexual pathway is retained in this species in conjunction with this finding indicates that sex may play an important role in the dynamics of *Hieracium pilosella* populations.

The finding that sexual reproduction is universal in apomictic populations of *Hieracium pilosella,* but that pathogens and parasites are seemingly almost completely absent in New Zealand, indicates that "Red Queen" theories (van Valen 1973, Bell 1982) are not an adequate explanation in this case. However due to the fact that the residual sexual reproduction may in *H. pilosella* be of minimal cost, due to the majority of offspring being produced via apomixis, the impact of such parasites or pathogens to maintain this reproductive mode could be relatively minor. The presence of such a parasite may not be immediately obvious, illustrated by the discovery of powdery mildew at the Chilton valley site only after visiting in late autumn in 2000. Up until this point it was not known that this pathogen was present in this area. However, the finding that there was no significant effect of the presence of powdery mildew on the reproductive mode of *H. pilosella,* indicates that this has most likely not been an important factor in selecting for the level of residual sexual reproduction since its' arrival in New Zealand. Due to the recent origin of *H. pilosella* in New Zealand, the number of generations since its' establishment have been limited. Therefore, it is not possible to exclude the prospect that the rates of sexual reproduction observed are not the effect of past selection by alternative pests or parasites in the native range.

The indications of a possible high mutation rate, based on the findings of the compatibility analysis (Chapter III), in *Hieracium pilosella* may be interesting in regard to the suggestion by Kondrashov (1982, 1988) that the accumulation of deleterious mutations may be responsible for the maintenance of sex. Although more recent works

have suggested that mutation alone is not a sufficient explanation, it is still included as a vital mechanism in many models (Howard & Lively 1994, Lively & Howard 1995, Burt 2000). However it is the accumulation of mutations that these theories rely on rather than a substantial mutation rate *per se*. As has been noted, species with the possibility to periodically undergo recombination or generate new clones can escape from this problem (de Kovel & de Jong 2001). Whether the low rates of recombination observed in *H. pilosella* can be sufficiently explained by the accumulation of mutations is unknown, however this is a potential area for future investigation.

Recent reviews have relied on modelling rather than the collection of empirical data (see Howard & Lively 1994, Lively & Howard 1995, Green & Noakes 1995, Hurst & Peck 1996, Peck *et al.* 1998, Burt 2000, Peck & Waxman 2000, Stauffer *et al.* 2000, Sasaki *et al.* 2002). Although the idea of using models to examine these ideas is attractive, it is possible that in the case of models for the maintenance of sex, more empirical data is required to better understand the range of systems being considered. Few models include taxa with mixed mating systems, usually only comparing obligate sexual and asexual lines (see Hurst & Peck 1996). It is also interesting to note that the majority of investigators use models based on animal species, to the point that one paper even reports that mixed systems are "remarkably elusive, particularly in animals" and states that if such a system did arise it would be a "transient state (presumably to obligate apomixis and extinction)" (Burt 2000). Unfortunately it seems that the idea of asexuals as an evolutionary "dead-end" is still a widely held belief, despite the numerous studies of facultative taxa, albeit predominantly plants (see Chapter 3). In contrast to this is the conclusion of Hurst and Peck (1996) (see also Green & Noakes 1995) who suggest that a low rate of recombination is sufficient to provide most of the benefits of sex, modelled as the removal of deleterious mutations, as long as the mutation rate is not "very high". It is also suggested that facultative sex may often lead to obligate sex as the ability to purge deleterious mutations leads to decreased proof reading or repair efficiency (Hurst & Peck 1996). The ability to remove deleterious mutations via recombination means that it is possible for an organism to gain efficiency by allocating less effort to these functions, as sex increases their ability to remove copy errors. The association between facultative apomixis and polyploidy could be explained in this framework due to the ability of polyploids to carry higher levels of deleterious mutations. Because they have several

copies of each allele at a locus, they are therefore able to survive decreased proof reading efficiency without requiring an increase in the frequency of sex.

VI.2 PRACTICAL APPLICATIONS

VI.2.1 APPLICATION OF ENVIRONMENTAL DATA TO THE APOMIXIS PROGRAMME FOR CROPPING.

The environment plays a significant role in explaining variation in the expression of apomixis in *Hieracium pilosella* growing under field conditions in New Zealand. It is clear, however, that the genotype of the accession in question is possibly of more importance than the environment in determining the levels of residual sexual reproduction. From these findings it seems imperative to identify the action of modifiers on the primary apomixis locus, as has been suggested in recent studies, to better understand the genetic nature of the variation in this trait (Koltunow *et al.* 2000, Bicknell *et al.* 2001). Additionally, this study has identified other facets of apomixis in *H. pilosella* that may not be desirable in crop species.

The role of the environment on the expression of apomixis is most likely due to changes in physiology triggered by environmental conditions. The effect of the environment on future-developed apomictic crop species may be able to be minimised by determining the action of these modifiers, and using promoter sequences to by-pass the action of any problematic loci. It is interesting to note, however, that the direction of the response to temperature and available water may be universal in most species, as this has already been observed in members of both the Asteraceae (*Hieracium pilosella*), and the Gramineae (see Knox 1967). The environmental control of the expression of apomixis as found in *H. pilosella* emphasises the need to develop crop genotypes that minimise the level of sexual reproduction over a range of environmental parameters.

Another potentially undesirable phenomenon to be addressed is the association between elevated levels of sexual reproduction and an increase in yield. This may prove problematic if this is found to occur universally in commercially developed apomictic species. The demonstration of this association identifies another potential issue to be addressed in the development of an apomictic crop species. The fact that evolutionary theory predicts that the cost to plant fitness will be minimised under these conditions may also indicate that this may be favoured. If this is the case then a trade-off will exist

between maximising yield (particularly for species where seed is the end product) and minimising the generation of diversity in the crop stand. Developing a genotype without the positive relationship between fecundity and residual sexual reproduction observed in *Hieracium pilosella* will be critical in the success of such programmes. Although there may be some scope for the manipulation of the management of crop stands to minimise the undesirable traits for cropping identified in *H. pilosella*, these will hopefully be able to be overcome by genetic engineering.

Although it has been suggested that a low rate of sexual reproduction may be desirable in apomictic crop species to allow the development of further strains (Koltunow *et al.* 1995), the frequencies observed in *Hieracium pilosella* may be too high for commercial purposes. The complex population structures of field populations of *H. pilosella* demonstrate that a level of recombination such as that recorded is sufficient to generate complex stands. The potential for the loss of hybrid vigour via recombination may reduce yields to the point where the use of this technology is not a significant improvement over conventional methods. The use of a genetic / physiological engineering approach may allow the use of inducible promoters, that are only expressed under specific conditions or developmental states, to facilitate facultative apomixis in crop species for breeding purposes (R.A. Bicknell *pers. comm.*). This will maintain the integrity of the crop strain under production conditions, and still retain flexibility in allowing future breeding from existing strains.

VI.2.2 IMPLICATIONS FOR BIO-CONTROL

The complex reproductive patterns observed, and the potential for micro-evolution in populations via recombination in facultative apomicts, make the application of specific bio-control organisms to *Hieracium pilosella* problematic. The lack of investigation into the genetic diversity in the host organism in some bio-control programmes to date has most likely led to the lack of successful control (Barrett 1992). The findings of this study indicate that bio-control agents must have a wide specificity to be successful, particularly over longer time periods. The differential infection of populations by the rust fungus

Puccinia hieracii var. *piloselloidarum* (Syrett *et al.* 2002) is indicative of a control agent with too much specificity to be universally effective. The success of the *Puccinia* sp. control of the apomict *Chondrilla juncea* is unlikely to be repeated on *Hieracium* spp. due to the complexity of the population structure and reproductive mode of the later. The very simple population structure of *C. juncea* made it amenable to such a scheme; in the case of *H. pilosella* the complexity of the population structures makes this a less viable strategy.

The current control programmes for *Hieracium* spp. in New Zealand utilise herbivorous or parasitic insects. Although the effect of one of these species alone may not be sufficient for successful control, the use of a suite of such insects with different target areas of the plants' reproductive structures may be more effective (Syrett *et al.* 2001). While most successful control programmes of plant species to date are based on fungal control agents, in the case of *H. pilosella* in New Zealand, an insect consortium may be most effective. Although the fungal approach has many advantages, unless a wide range of effective isolates to cover the genetic diversity of the host are found, this may not be the most practical approach. The potential rapidity for change in *H. pilosella* populations demands more generalist agents than rust fungi, regardless of the diversity of isolates available and the potential for recombination in this group (Jenkins 1995). This is not to say that other fungal pathogens, with wider specificity than the highly conserved *Puccinia* spp., would not be potential candidates as control agents, although the selection of a suite of strains, and a group with an open recombination system, would be more productive.

The discovery of obligate sexual plants in the high country of New Zealand may also further complicate the successful control of this species by biological agents. The greater potential for short-term evolution that these plants represent can result in even more complex population structures (Chapman *et al. in prep*) and the faster spread of resistance to control agents.

VI.3 FUTURE DIRECTIONS

Due to the two-fold potential applications of the data from this investigation, future research can be targeted to different problems. While some are of most interest to those looking at utilising a gene or genes from *Hieracium pilosella* for transfer of apomixis to crop plants, others are more focussed on the evolutionary potential of facultative apomictic species. Some potential areas for future study resulting from this investigation are as follows:

- A study of the effect of commonly applied phytohormone synthetics on the expression of apomixis in this group may identify possible methods for the manipulation of the reproductive mode of this species. The identification of a physiological mechanism may be more amenable to manipulation than the environmental parameters identified, and this work would also contribute to a greater understanding of apomixis in *Hieracium pilosella.*

- The identification and characterisation of the modifiers to the primary apomixis locus and how these interact with the environment will also facilitate the understanding of the differential expression of this trait in *Hieracium pilosella.* Better understanding of this may be necessary to de-couple the positive relationship observed in this study between seed production and the frequency of sexual reproduction.

- Further investigation of potential genetic and biotic factors promoting residual sexual reproduction in *Hieracium pilosella.* The use of this plant in as a model species for such investigations or the construction of models from demographic data may provide a very useful system for the investigation of the theories for the maintenance of sex.

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APPENDICES

APPENDIX 1.1 FIELD SITE DESCRIPTIONS.

Field site parameters:

(All co-ordinate data from a Garmin GPS 12 Global Positioning System, Version 4.58, Garmin Corporation, Olathe, KS USA 66062).

1). Cave stream: S 43 10.960' E 171 44.350' ~ 700M alt.

The site has a Southerly aspect and is formed from a river flood plain. This site has been free from grazing by all mammalian species since 1994, due to its status as a protected plant regeneration area by the Department of Conservation. Situated on a limestone base (Castle Hill assemblage) with well-drained soil and low to moderate grass cover. Moisture levels are extremely variable. During early November 1998, the site was boggy in places with moist conditions prevalent. By mid January 1999, drought conditions existed at the site, with most vegetation, including *Hieracium pilosella* burnt off. The summer of 1999 / 2000 produced no viable inflorescences due to extreme drought conditions. Abortion of *H. pilosella* inflorescences was frequent in all seasons. *H. pilosella* and *H. praealtum* are both present in high densities at this site. The vegetation has been moderately modified by farming practices and is in a state of regeneration.

2). *Dracophyllum* flat lower: S 43 09.005' E 171 44.048' ~ 790M alt.

This site has a high proportion of shrub cover, and hardly any grass species present, unlike all of the other sites. At ground level, however, there are very low levels of cover. The site has moderate moisture levels, but is flooded in early summer each year. Dominant species are *Dracophyllum pronum, D. uniflorum, D. longiflorum, Leptospermum scoparium* and seedlings of *Nothofagus solandri.* High densities of *Hieracium* spp. (*H. pilosella, H. praealtum, H.* x*stoloniflorum,* and *H. lepidulum*) exist and this appears to be late flowering due to the flooding present. Invasion by wilding pines (*Pinus radiata*) also occurs at this site from neighbouring exotic plantations, but otherwise it mostly contains native vegetation. Some evidence of hare (*Lepus europaeus*) grazing is present in the area. No other grazing mammals are present. East to Southeast aspect and on remnant beech forest soils.

3). *Dracophyllum* Flat upper: S 43 09.005' E 171 44.047' ~ 810M alt.
This site is situated in a small gully, and has a high proportion of shrub cover. *Hieracium xstoloniflorum* is present at high densities, *H. pilosella* being the only species at higher densities at this site. The site has moderate moisture levels, but lacks the flooding of the other site at this location. The base is beech forest soil, and the dominant species are *Dracophyllum pronum, D. uniflorum, D. longiflorum, Discaria toumatou, Leptospermum scoparium* and seedlings of *Nothofagus solandri. H. pilosella* flowers earlier than the lower site, and flowering is more synchronous and therefore over a shorter time. Other site parameters are as for the lower site.

4). Cass flats: S 43 02.173' E 171 45.691' ~ 580M alt.
High densities of grass cover are present at this site, both exotic and native. Species that are present include *Poa cita, P. colensoi, Festuca novaezealandiae,* and the exotics *Agrostis capillaris* and *Anthoxanthum odoratum.* Also present at the site is *Hypochoeris radicata.* Due to the management practices at the University of Canterbury Cass reserve, this site had to be fenced to keep sheep (*Ovis aries*) out of the experimental area, as they preferentially graze *H. pilosella* inflorescences. Hares (*Lepus europaeus*) are present and are not excluded by the existing fences. Ground cover is very dense, and *Hieracium pilosella* is restricted to areas that are disturbed such as vehicle and stock tracks. *H. pilosella* is at lower densities than all of the other sites examined. The site has a Nor-west aspect and is on beech forest soils.

5). Chilton valley: S 42 02.223' E 171 46.336' ~ 740M alt.
This site has the most shelter from prevailing winds and experiences high levels of precipitation and light. It is situated in the valley between Cass hill and Sugarloaf at the University of Canterbury Cass reserve. The soil has a different composition to that on the flat, with better drainage and thicker topsoil. *Hieracium pilosella* is at moderate densities, other common species at the site being *Discaria toumatou, Agrostis capillaris* and *Poa cita.* Temperature is more extreme than the lowland site due to the sheltered aspect. Ground cover is extensive although *H. pilosella* has been a very successful invader into the existing sward. Again, this site is fenced to keep grazing stock out, but

hares (*Lepus europaeus*) and opossum (*Trichosurus vulpecula*) are present. Nor-West aspect and beech forest soils.

6). Little river: S 43 66.100' E 172 46.500' ~ 650M alt

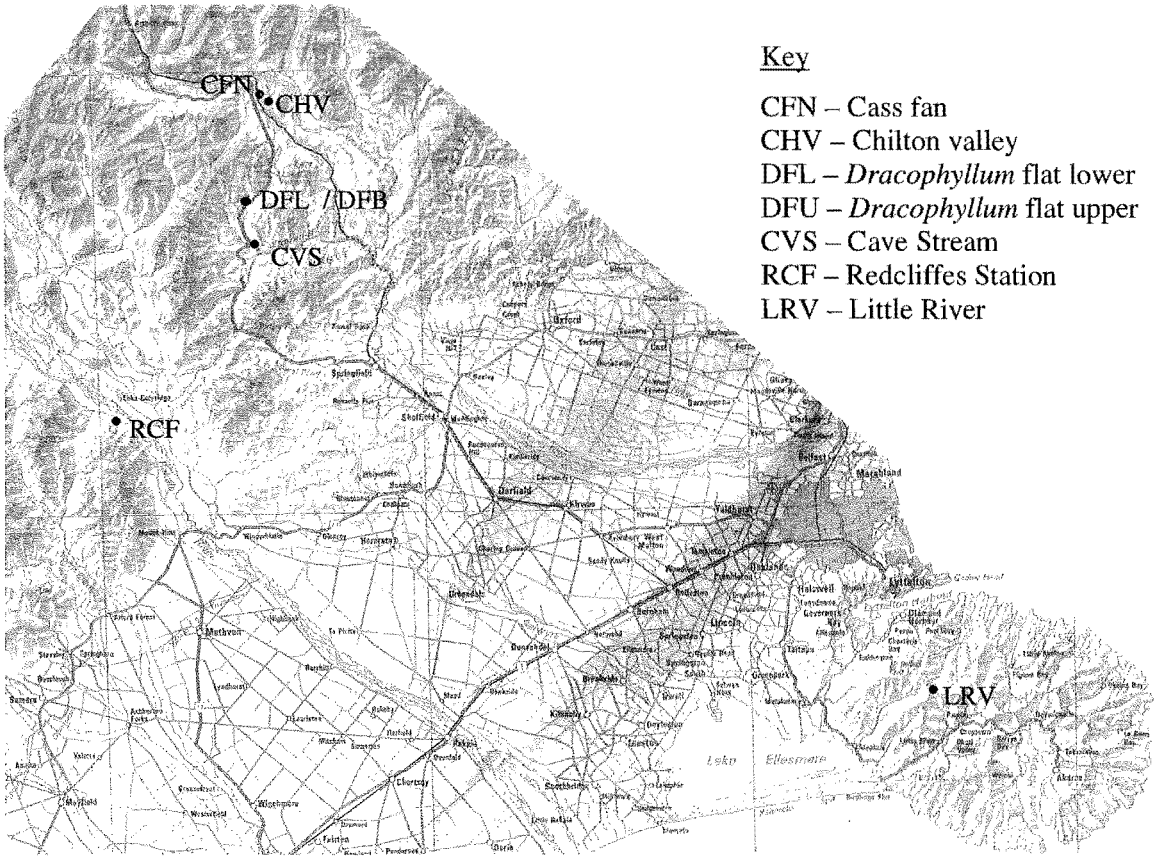
Modified farmland site with relatively heavy grazing. High densities of *Hieracium pilosella* are present, but with a patchy distribution. Cages are used over the plants to keep stock out, and these also exclude other mammalian pest species. This allowed the otherwise low grass cover to increase. The site experiences high rainfall, and is exposed to high winds. Highly modified compared to other sites due to pasture grass and grazing animals being present year round, with hardly any endemic species present. The dominant species are *Anthoxanthum odoratum* and *Agrostis capillaris.* Soil type is also different to the high country sites, with the base of volcanic origin. Temperature conditions are different to the inland sites with coastal weather patterns and rainfall is substantially higher than all other sites. South-eastern aspect.

7). Redcliffes station: S 43° 23.156' E 171° 31.027' ~600M alt

The most southern of the sites, situated on the south bank (true right) of the Rakaia river. Site has a high density of grass cover, and fertiliser application is a feature of the stations management. *Hieracium pilosella* is mostly restricted to the margins of *Chionochloa* spp. and *Discaria toumatou.* Both powdery mildew (*Erysiphe* spp.) and rust fungus (*Puccinia hieracii* var. *piloselloidarum*) are present. Cages are used over the plants to keep stock out, and these also exclude other mammalian pest species. The site is exposed to extremes of temperature and moisture. The dominant species are *Anthoxanthum odoratum* and *Agrostis capillaris*. Northern aspect.

APPENDIX 1.2

Figure A 1.2 Map of field site locations.



Scale approximately 1: 500 000, NZMS 242 – sheet 3.

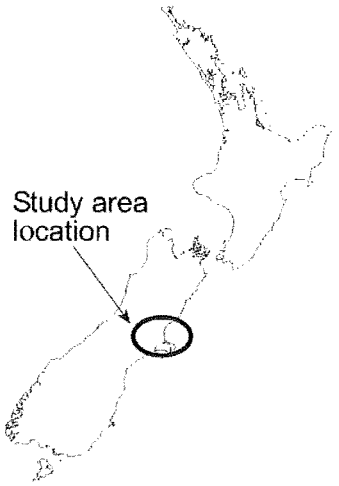


Table A 2.1.1 1998 / 1999 field season: pollination dates and reproductive patterns

	Cave Stream																
code no.	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	Ploidy level
1001	7/01/1999	11/01/1999	28/01/1999	81	7	59		72.84	74	79.73	20/05/1999	12/08/1999	14/10/1999	59	0	0.00	
1002	7/01/1999	11/01/1999	28/01/1999	129	15	77		59.69	114	67.54	20/05/1999	1/09/1999	11/10/1999	52	0	0.00	
1003	7/01/1999	11/01/1999	28/01/1999	75	10	46		61.33	65	70.77	20/05/1999	27/09/1999	16/11/1999	46	0	0.00	
1004	7/01/1999	10/01/1999	28/01/1999	91	8	33		36.26	83	39.76	20/05/1999	16/07/1999	20/08/1999	32	1	3.13	
1005	7/01/1999	10/01/1999	28/01/1999	132	8	93		70.45	124	75.00	20/05/1999	13/09/1999	6/11/1999	92	0	0.00	36/30
1006	7/01/1999	11/01/1999	28/01/1999	102	22	34		33.33	80	42.50	20/05/1999	11/10/1999	17/11/1999	33	0	0.00	
1007	7/01/1999	10/01/1999	28/01/1999	90	9	57		63.33	81	70.37	20/05/1999	13/09/1999	1/11/1999	57	0	0.00	
1008	7/01/1999	10/01/1999	28/01/1999	104	2	63		60.58	102	61.76	20/05/1999	15/09/1999	10/11/1999	63	0	0.00	
1009	7/01/1999	11/01/1999	28/01/1999	90	17	45		50.00	73	61.64	20/05/1999	27/09/1999	16/11/1999	45	0	0.00	
1010	7/01/1999	11/01/1999	28/01/1999	116	8	63		54.31	108	58.33	20/05/1999	27/09/1999	16/11/1999	63	0	0.00	
1011	7/01/1999	10/01/1999	28/01/1999	70	13	44		62.86	57	77.19	20/05/1999	16/07/1999	23/08/1999	44	0	0.00	
			total seeds	1080	119	614		56.85	961	63.89				586	1	0.17	
			mean	98.182	10.818	55.818		56.817	87.364	64.055				53.273	0.091	0.284	
			std dev	20.668	5.528	18.154		12.614	21.477	13.168				16.758	0.302	0.942	

NB// "infected refers to whether there was any fungal growth in the culture during germination.

	Dracophyllum flat upper																
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	Ploidy level
1015	7/01/1999	9/01/1999	28/01/1999	109	9	67		61.47	100	67.00	1/04/1999	20/05/1999	31/07/1999	59	3	5.08	36
1016	7/01/1999	9/01/1999	28/01/1999	95	12	41		43.16	83	49.40	1/04/1999	29/06/1999	18/08/1999	40	0	0.00	
1017	7/01/1999	9/01/1999	28/01/1999	114	4	72		63.16	110	65.45	1/04/1999	24/06/1999	6/09/1999	72	0	0.00	45
1021	7/01/1999	10/01/1999	28/01/1999	59	14	34		57.63	45	75.56	1/04/1999	21/05/1999	31/07/1999	31	0	0.00	
1022	7/01/1999	11/01/1999	28/01/1999	79	5	55		69.62	74	74.32	1/04/1999	6/09/1999	25/10/1999	49	0	0.00	
1023	7/01/1999	11/01/1999	28/01/1999	114	11	28		24.56	103	27.18	2/08/1999	13/09/1999	1/11/1999	9	0	0.00	
1024	7/01/1999	11/01/1999	28/01/1999	98	10	37	y	37.76	88	42.05	2/08/1999	1/09/1999	23/10/1999	35	1	2.86	45
1025	7/01/1999	11/01/1999	28/01/1999	109	14	43	y	39.45	95	45.26	2/08/1999	20/08/1999	22/09/1999	41	1	2.44	45
			total seeds	573	58	377		65.79	698	54.01				336	5	1.49	
			mean	97.125	9.875	47.125		49.600	87.250	55.778				42.000	0.625	1.298	
			std dev	19.416	3.758	15.905		15.592	20.590	17.370				18.906	1.061	1.946	

	Dracophyllum flat lower																
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	Ploidy level
1012	7/01/1999	9/01/1999	28/01/1999	82	25	41		50.00	57	71.93	1/04/1999	21/05/1999	17/06/1999	39	34	87.18	36
1013	7/01/1999	9/01/1999	28/01/1999	80	4	52		0.65	76	0.68	1/04/1999	1/09/1999	19/10/1999	50	0	0.00	37
1014	7/01/1999	9/01/1999	28/01/1999	120	11	69		0.58	109	0.63	1/04/1999	2/08/1999	17/09/1999	67	7	10.45	45
1018	7/01/1999	10/01/1999	28/01/1999	90	28	57		0.63	62	0.92	1/04/1999	24/06/1999	10/09/1999	57	35	61.40	36
1019	7/01/1999	10/01/1999	28/01/1999	73	41	30		0.41	32	0.94	1/04/1999	9/06/1999	9/08/1999	29	12	41.38	36
1020	7/01/1999	10/01/1999	28/01/1999	104	6	72		0.69	98	0.73	1/04/1999	24/06/1999	8/09/1999	70	0	0.00	
1026	7/01/1999	11/01/1999	28/01/1999	80	6	33	y	0.41	74	0.45	2/08/1999	20/08/1999	9/10/1999	33	0	0.00	
1027	7/01/1999	11/01/1999	28/01/1999	113	8	64		0.57	105	0.61	2/08/1999	18/10/1999	12/12/1999	63	0	0.00	
			total seeds	460	89	418		53.94	613	76.89				408	88	21.57	
			mean	92.750	16.125	52.250		6.743	76.625	9.612				51.000	11.000	25.051	
			std dev	17.393	13.538	16.140		17.479	26.479	25.181				15.811	15.147	34.167	

Table A 2.1.1 cont.

	Cass flats																
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	Ploidy level
1028	6/01/1999	8/01/1999	28/01/1999	66	16	36		54.55	50	72.00	2/08/1999	31/08/1999	9/10/1999	36	0	0.00	
1029	6/01/1999	8/01/1999	28/01/1999	78	31	37		47.44	47	78.72	2/08/1999	12/10/1999	26/11/1999	27	3	11.11	
1030	6/01/1999	8/01/1999	28/01/1999	100	21	69		69.00	79	87.34	2/08/1999	12/10/1999	26/11/1999	57	4	7.02	
1031	6/01/1999	8/01/1999	28/01/1999	82	22	54		65.85	60	90.00	2/08/1999	18/10/1999	13/12/1999	53	6	11.32	
1032	6/01/1999	8/01/1999	28/01/1999	89	28	21	y	23.60	61	34.43	2/08/1999	27/09/2009	16/11/1999	6	0	0.00	
1033	6/01/1999	8/01/1999	28/01/1999	70	20	23		32.86	50	46.00	2/08/1999	12/10/1999	25/11/1999	11	1	9.09	
1034	6/01/1999	8/01/1999	28/01/1999	92	25	36		39.13	67	53.73	30/09/1999	18/11/1999	13/12/1999	32	0	0.00	
1035	6/01/1999	8/01/1999	28/01/1999	83	18	20	y	24.10	65	30.77	26/03/1999	26/04/1999	2/06/1999	20	0	0.00	
1036	6/01/1999	8/01/1999	28/01/1999	99	19	39	y	39.39	80	48.75	26/03/1999	13/04/1999	16/07/1999	34	4	11.76	
1037	6/01/1999	9/01/1999	28/01/1999	65	31	3		4.62	34	8.82	26/03/1999	21/05/1999	17/06/1999	3	1	33.33	36
1038	6/01/1999	9/01/1999	28/01/1999	119	19	73		61.34	100	73.00	26/03/1999	26/04/1999	23/06/1999	72	4	5.56	45
1039	6/01/1999	9/01/1999	28/01/1999	78	29	22	y	28.21	49	44.90	26/03/1999	6/04/1999	17/05/1999	4	0	0.00	45
1040	6/01/1999	9/01/1999	28/01/1999	82	25	46		56.10	57	80.70	26/03/1999	12/08/1999	16/10/1999	46	4	8.70	34
1041	6/01/1999	9/01/1999	28/01/1999	77	21	56		72.73	56	100.00	26/03/1999	17/07/1999	30/08/1999	56	4	7.14	36
1042	6/01/1999	9/01/1999	28/01/1999	92	29	36	y	39.13	63	57.14	26/03/1999	26/04/1999	2/06/1999	30	2	6.67	45
1043	6/01/1999	11/01/1999	28/01/1999	73	21	36		49.32	52	69.23	26/03/1999	11/08/1999	25/09/1999	36	1	2.78	45
1044	6/01/1999	11/01/1999	28/01/1999	61	22	35		57.38	39	89.74	26/03/1999	31/08/1999	11/10/1999	34	1	2.94	
1045	6/01/1999	11/01/1999	28/01/1999	80	25	28		35.00	55	50.91	26/03/1999	26/04/1999	17/06/1999	28	0	0.00	45
			total seeds	1486	422	670		45.09	1064	62.97				585	35	5.98	
			mean	82.556	23.444	37.222		44.429	59.111	62.011				32.500	1.944	6.523	
			std dev	14.345	4.617	17.599		18.132	15.628	24.308				19.410	1.955	7.990	
	Chilton valley																
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	Ploidy level
1046	6/01/1999	8/01/1999	28/01/1999	58	50	0		0.00	8	0.00	21/06/1999	X	X	0	0	na	
1047	6/01/1999	8/01/1999	28/01/1999	66	6	21		31.82	60	35.00	21/06/1999	19/08/1999	25/09/1999	20	0	0.00	
1048	6/01/1999	8/01/1999	28/01/1999	96	27	0	y	0.00	69	0.00	21/06/1999	X	X	0	0	na	
1049	6/01/1999	8/01/1999	28/01/1999	97	11	2		2.06	86	2.33	21/06/1999	27/09/1999	16/11/1999	2	0	0.00	
1050	6/01/1999	8/01/1999	28/01/1999	79	22	22		27.85	57	38.60	21/06/1999	12/10/1999	25/11/1999	22	1	4.55	
1051	6/01/1999	8/01/1999	28/01/1999	106	28	29		27.36	78	37.18	21/06/1999	12/10/1999	25/11/1999	29	0	0.00	
1052	6/01/1999	8/01/1999	28/01/1999	65	14	22		33.85	51	43.14	21/06/1999	19/08/1999	14/10/1999	22	0	0.00	
1053	6/01/1999	8/01/1999	28/01/1999	82	16	59		71.95	66	89.39	21/06/1999	12/08/1999	20/12/1999	28	0	0.00	
1054	6/01/1999	8/01/1999	28/01/1999	69	13	23		33.33	56	41.07	21/06/1999	12/08/1999	22/09/1999	16	0	0.00	
1055	6/01/1999	8/01/1999	28/01/1999	71	15	29		40.85	56	51.79	21/06/1999	11/10/1999	20/11/1999	27	0	0.00	
1056	6/01/1999	10/01/1999	28/01/1999	78	17	26		33.33	61	42.62	21/06/1999	11/10/1999	25/11/1999	26	0	0.00	
1057	6/01/1999	10/01/1999	28/01/1999	70	13	22		31.43	57	38.60	30/09/1999	18/11/1999	22/12/1999	22	0	0.00	
1058	6/01/1999	10/01/1999	28/01/1999	61	14	42		68.85	47	89.36	30/09/1999	11/10/1999	22/11/1999	41	0	0.00	
1059	6/01/1999	10/01/1999	28/01/1999	108	30	66		61.11	78	84.62	24/03/1999	20/05/1999	22/07/1999	65	1	1.54	
1060	6/01/1999	10/01/1999	28/01/1999	77	15	49		63.64	62	79.03	24/03/1999	1/09/1999	23/10/1999	49	0	0.00	36
1061	8/01/1999	10/01/1999	28/01/1999	84	17	51		60.71	67	76.12	24/03/1999	20/08/1999	29/09/1999	51	0	0.00	
1062	8/01/1999	10/01/1999	28/01/1999	83	8	77		92.77	75	102.67	24/03/1999	29/08/1999	16/08/1999	77	2	2.60	
1063	8/01/1999	10/01/1999	28/01/1999	82	17	24		29.27	65	36.92	24/03/1999	17/06/1999	9/08/1999	24	0	0.00	
1064	8/01/1999	11/01/1999	28/01/1999	77	17	42		54.55	60	70.00	24/03/1999	26/04/1999	4/06/1999	41	0	0.00	45
1065	8/01/1999	11/01/1999	28/01/1999	80	21	43		53.75	59	72.88	24/03/1999	2/08/1999	30/08/1999	43	0	0.00	
1066	8/01/1999	11/01/1999	28/01/1999	79	23	32	y	40.51	56	57.14	24/03/1999	26/04/1999	4/06/1999	32	0	0.00	
1067	8/01/1999	11/01/1999	28/01/1999	58	5	36		62.07	53	67.92	24/03/1999	9/06/1999	9/08/1999	35	0	0.00	
1068	8/01/1999	11/01/1999	28/01/1999	89	13	52		58.43	76	68.42	24/03/1999	24/06/1999	11/09/1999	51	0	0.00	45
1069	8/01/1999	11/01/1999	28/01/1999	123	35	71		57.72	88	80.68	24/03/1999	26/04/1999	27/05/1999	71	1	1.41	36
			total seeds	1938	447	840		43.34	1491	56.34				794	5	0.630	
			mean	80.750	18.625	35.000		43.217	62.125	54.395				33.083	0.208	0.459	
			std dev	16.192	9.942	21.028		23.493	15.807	28.733				20.545	0.509	1.137	

Table A 2.1.1 cont.

	Little river																
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	Ploidy level
1070	16/01/1999	19/01/1999	9/02/1999	72	4	45		62.50	68	66.18	30/09/1999	27/10/1999	20/12/1999	45	2	4.44	
1071	16/01/1999	19/01/1999	9/02/1999	78	3	40		51.28	75	53.33	30/09/1999	22/11/1999	6/01/1999	39	0	0.00	
1072	16/01/1999	19/01/1999	9/02/1999	91	6	59		64.84	85	69.41	30/09/1999	22/11/1999	20/01/2000	59	1	1.69	
1073	16/01/1999	19/01/1999	9/02/1999	89	4	31	y	34.83	85	36.47	30/09/1999	27/10/1999	13/12/1999	31	2	6.45	
1074	16/01/1999	19/01/1999	9/02/1999	83	13	30		36.14	70	42.86	30/09/1999	22/11/1999	6/01/1999	30	0	0.00	
1075	16/01/1999	19/01/1999	9/02/1999	86	5	13		15.12	81	16.05	30/09/1999	22/11/1999	6/01/1999	13	1	7.69	
1076	16/01/1999	19/01/1999	9/02/1999	87	3	43		49.43	84	51.19	30/09/1999	18/11/1999	22/12/1999	40	2	5.00	
1077	16/01/1999	19/01/1999	9/02/1999	86	6	49		56.98	80	61.25	30/09/1999	18/11/1999	21/01/2000	48	1	2.08	
1078	16/01/1999	19/01/1999	9/02/1999	84	11	24	y	28.57	73	32.88	14/10/1999	1/11/1999	20/12/1999	24	0	0.00	
1079	16/01/1999	19/01/1999	9/02/1999	86	3	50		58.14	83	60.24	14/10/1999	26/11/1999	6/01/1999	41	3	7.32	
1080	16/01/1999	19/01/1999	9/02/1999	91	5	28	y	30.77	86	32.56	14/10/1999	1/11/1999	22/12/1999	28	2	7.14	
1081	16/01/1999	25/01/1999	9/02/1999	77	5	59		76.62	72	81.94	14/10/1999	18/11/1999	19/01/2000	59	0	0.00	
1082	16/01/1999	19/01/1999	15/02/1999	82	5	59		71.95	77	76.62	14/10/1999	26/11/1999	24/01/2000	59	1	1.69	
1083	16/01/1999	19/01/1999	15/02/1999	74	4	38		51.35	70	54.29	14/10/1999	13/12/1999	28/01/2000	34	0	0.00	
1084	16/01/1999	19/01/1999	15/02/1999	100	22	46		46.00	78	58.97	14/10/1999	13/12/1999	26/01/2000	45	0	0.00	
1085	16/01/1999	19/01/1999	15/02/1999	78	9	42		53.85	69	60.87	14/10/1999	1/12/1999	28/01/2000	40	0	0.00	
1086	16/01/1999	19/01/1999	15/02/1999	62	5	29		46.77	57	50.88	14/10/1999	13/12/1999	24/01/2000	29	1	3.45	
1087	16/01/1999	25/01/1999	15/02/1999	91	7	57		62.64	84	67.86	14/10/1999	18/11/1999	12/01/2000	56	0	0.00	
1088	16/01/1999	25/01/1999	15/02/1999	89	16	55		61.80	73	75.34	14/10/1999	25/11/1999	6/01/1999	55	0	0.00	
1089	16/01/1999	25/01/1999	15/02/1999	81	22	0	y	0.00	59	0.00	2/11/1999	X	X	0	0	na	
1090	16/01/1999	25/01/1999	15/02/1999	61	17	39		63.93	44	88.64	2/11/1999	15/12/1999	28/01/2000	39	1	2.56	
1091	25/01/1999	2/02/1999	19/02/1999	81	11	74		91.36	70	105.71	2/11/1999	13/12/1999	26/01/2000	73	2	2.74	
1092	25/01/1999	2/02/1999	19/02/1999	100	17	53		53.00	83	63.86	23/03/1999	21/04/1999	2/06/1999	51	2	3.92	45
1093	25/01/1999	2/02/1999	19/02/1999	96	7	35	y	36.46	89	39.33	23/03/1999	6/04/1999	14/06/1999	33	2	6.06	45
1094	25/01/1999	2/02/1999	19/02/1999	86	7	21	y	24.42	79	26.58	23/03/1999	12/04/1999	29/06/1999	21	0	0.00	45
1095	25/01/1999	2/02/1999	19/02/1999	83	16	33		39.76	67	49.25	23/03/1999	21/04/1999	2/06/1999	30	2	6.67	45
1096	25/01/1999	2/02/1999	19/02/1999	83	4	39		46.99	79	49.37	23/03/1999	21/04/1999	2/06/1999	33	3	9.09	36
1097	25/01/1999	2/02/1999	19/02/1999	91	14	52		57.14	77	67.53	23/03/1999	2/08/1999	22/09/1999	52	2	3.85	
1098	2/02/1999	3/02/1999	4/02/1999	66	14	30		45.45	52	57.69	23/03/1999	15/06/1999	12/08/1999	30	0	0.00	
1099	2/02/1999	3/02/1999	4/02/1999	77	17	43		55.84	60	71.67	23/03/1999	12/04/1999	5/07/1999	39	2	5.13	45
1100	2/02/1999	3/02/1999	4/02/1999	67	6	34		50.75	61	55.74	23/03/1999	12/04/1999	12/07/1999	34	4	11.76	36
1101	2/02/1999	3/02/1999	4/02/1999	76	26	39	y	51.32	50	78.00	23/03/1999	12/04/1999	14/07/1999	36	0	0.00	
1102	2/02/1999	3/02/1999	4/02/1999	74	7	47		63.51	67	70.15	11/03/1999	25/03/1999	19/04/1999	36	0	0.00	
1103	2/02/1999	3/02/1999	4/02/1999	62	14	12	y	19.35	48	25.00	11/03/1999	26/03/1999	23/04/1999	7	0	0.00	45
1104	2/02/1999	3/02/1999	4/02/1999	56	3	31		55.36	53	58.49	11/03/1999	26/03/1999	23/04/1999	20	1	5.00	36
1105	2/02/1999	3/02/1999	4/02/1999	80	12	41		51.25	68	60.29	11/03/1999	25/03/1999	15/04/1999	41	1	2.44	36
1106	2/02/1999	3/02/1999	4/02/1999	70	15	49		70.00	55	89.09	11/03/1999	26/03/1999	23/04/1999	31	3	9.68	36
1107	2/02/1999	3/02/1999	4/02/1999	89	12	30	y	33.71	77	38.96	11/03/1999	26/03/1999	15/04/1999	23	0	0.00	
1108	2/02/1999	3/02/1999	4/02/1999	91	11	50		54.95	80	62.50	11/03/1999	22/03/1999	19/04/1999	23	0	0.00	45
1109	2/02/1999	3/02/1999	4/02/1999	74	9	49		66.22	65	75.38	11/03/1999	22/03/1999	23/04/1999	19	0	0.00	
1110	2/02/1999	3/02/1999	4/02/1999	65	12	37		56.92	53	69.81	11/03/1999	22/03/1999	23/04/1999	27	1	3.70	45
1111	2/02/1999	3/02/1999	4/02/1999	61	11	25	y	40.98	50	50.00	11/03/1999	22/03/1999	23/04/1999	25	1	4.00	36
1112	2/02/1999	3/02/1999	4/02/1999	57	14	9	y	15.79	43	20.93	11/03/1999	22/03/1999	23/04/1999	5	0	0.00	45
1113	15/02/1999	19/02/1999	19/03/1999	55	7	27		49.09	48	56.25	2/11/1999	13/12/1999	24/01/2000	27	0	0.00	
1114	15/02/1999	19/02/1999	19/03/1999	51	11	22		43.14	40	55.00	2/11/1999	15/12/1999	26/01/2000	22	0	0.00	
1115	15/02/1999	19/02/1999	19/03/1999	54	0	35		64.81	54	64.81	2/11/1999	15/12/1999	28/01/2000	35	0	0.00	
1116	15/02/1999	19/02/1999	19/03/1999	61	6	14	y	22.95	55	25.45	2/11/1999	16/11/1999	12/01/2000	11	0	0.00	
1117	15/02/1999	19/02/1999	19/03/1999	53	10	17		32.08	43	39.53	2/11/1999	15/12/1999	26/01/2000	17	1	5.88	
1118	15/02/1999	19/02/1999	19/03/1999	68	2	58		85.29	66	87.88	2/11/1999	26/01/2000	29/02/2000	51	0	0.00	
1119	15/02/1999	19/02/1999	19/03/1999	19	3	10		52.63	16	62.50	2/11/1999	15/12/1999	24/01/2000	10	1	10.00	
			total seeds	3774	473	1852		49.07	3301	56.10				1676	45	2.68	
			mean	75.480	9.460	37.040		49.081	66.020	56.292				33.520	0.900	2.846	
			std dev	15.387	5.807	15.597		18.015	15.393	20.645				15.550	1.055	3.344	

Table A 2.1.2, 1999 / 2000 Field season: pollination dates and reproductive output

Rakaia Gorge

code n	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
2001	24/12/1999	30/12/1999	25/01/2000	56	43	11		19.64	13	84.62	1/02/2000	29/02/2000	29/03/2000	6	5	83.33	4.00	0.800	36
2002	24/12/1999	30/12/1999	25/01/2000	62	51	7	y	11.29	11	63.64	1/02/2000	12/02/2000	29/03/2000	7	7	100.00	3.00	0.429	36
2003	24/12/1999	30/12/1999	25/01/2000	76	57	12	y	15.79	19	63.16	1/02/2000	12/02/2000	29/03/2000	10	8	80.00	4.00	0.500	36
2004	24/12/1999	30/12/1999	25/01/2000	71	65	1	y	1.41	6	16.67	21/03/2000	20/04/2000	X	0	0	na	na	na	na
2005	24/12/1999	30/12/1999	25/01/2000	62	45	4		6.45	17	23.53	21/03/2000	20/04/2000	17/05/2000	2	2	100.00	0.00	0.000	36
2006	24/12/1999	30/12/1999	25/01/2000	64	35	25		39.06	29	86.21	21/03/2000	10/04/2000	20/06/2000	8	1	12.50	0.00	0.000	36
2007	24/12/1999	6/01/1999	25/01/2000	61	58	2		3.28	3	66.67	21/03/2000	26/04/2000	15/05/2000	1	0	0.00	na	na	36
2008	24/12/1999	6/01/1999	25/01/2000	23	9	13		56.52	14	92.86	21/03/2000	3/04/2000	9/05/2000	7	7	100.00	3.00	0.429	36
			total seeds	475	363	75		15.79	112	66.96				41	30	73.17	14.00	2.157	
			mean	59.375	45.375	9.375		19.181	14.000	62.167				5.125	3.750	67.976	2.333	0.360	
			std dev	15.964	17.517	7.800		19.252	8.053	28.310				3.643	3.370	43.119			

NB// "infected refers to whether there was any fungal growth in the culture during germination.

***Dracophyllum* flat lower**

	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
2021	3/12/1999	6/12/1999	10/01/2000	66	66	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2022	3/12/1999	6/12/1999	10/01/2000	81	71	0		0.00	10	0.00	27/03/2000	X	X	0	0	na	na	na	na
2023	3/12/1999	6/12/1999	10/01/2000	79	76	0		0.00	3	0.00	12/04/2000	X	X	0	0	na	na	na	na
2024	3/12/1999	9/12/1999	10/01/2000	74	74	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2025	3/12/1999	9/12/1999	10/01/2000	96	96	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2026	3/12/1999	9/12/1999	10/01/2000	86	86	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2027	3/12/1999	10/12/1999	10/01/2000	72	72	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2028	3/12/1999	10/12/1999	10/01/2000	79	79	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2029	3/12/1999	10/12/1999	10/01/2000	79	79	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2030	3/12/1999	10/12/1999	10/01/2000	75	75	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2031	3/12/1999	10/12/1999	10/01/2000	119	119	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2032	10/01/2000	15/01/2000	4/02/2000	68	60	7		10.29	8	87.50	12/04/2000	4/05/2000	27/06/2000	6	6	100.00	4.00	0.6667	36
2033	10/01/2000	17/01/2000	9/03/2000	76	42	27		35.53	34	79.41	12/04/2000	9/05/2000	6/07/2000	2	2	100.00	0.00	0.0000	36
			total seeds	1050	995	34		3.24	55	61.82				8	8	100.00	4	0.6667	
			mean	80.769	76.538	2.615		3.525	4.231	41.728				0.615	0.615	100.000	2.000	0.3333	
			std dev	13.815	18.077	7.576		10.027	9.558	48.296				1.710	1.710	0.000			

***Dracophyllum* flat upper**

	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
2051	3/12/1999	6/12/1999	10/01/2000	97	42	32	y	32.99	55	58.18	19/01/2000	27/01/2000	28/03/2000	10	0	0.000	na	na	46.26
2052	3/12/1999	6/12/1999	10/01/2000	85	29	22	y	25.88	56	39.29	19/01/2000	27/01/2000	28/03/2000	4	0	0.000	na	na	46.94
2053	3/12/1999	6/12/1999	10/01/2000	90	32	43	y	47.78	58	74.14	19/01/2000	27/01/2000	20/03/2000	43	1	2.326	0.00	0.000	46.57
2054	3/12/1999	6/12/1999	10/01/2000	119	41	69	y	57.98	78	88.46	1/02/2000	7/02/2000	24/03/2000	69	0	0.000	na	na	45.94
2055	3/12/1999	6/12/1999	10/01/2000	83	28	54		65.06	55	98.18	1/02/2000	9/03/2000	4/04/2000	52	0	0.000	na	na	na
2056	3/12/1999	7/12/1999	10/01/2000	97	59	1		1.03	38	2.63	1/02/2000	7/02/2000	X	0	0	na	na	na	na
2057	3/12/1999	7/12/1999	10/01/2000	100	36	64		64.00	64	100.00	22/02/2000	17/03/2000	17/04/2000	64	4	6.250	1.00	0.250	46.55
2058	3/12/1999	9/12/1999	10/01/2000	82	17	21	y	25.61	65	32.31	22/02/2000	6/03/2000	26/04/2000	8	0	0.000	na	na	46.93
2059	3/12/1999	9/12/1999	10/01/2000	68	19	12	y	17.65	49	24.49	22/02/2000	8/03/2000	11/04/2000	0	0	na	na	na	na
2060	3/12/1999	9/12/1999	10/01/2000	77	29	44	y	57.14	48	91.67	22/02/2000	6/03/2000	11/04/2000	44	0	0.000	na	na	46.78
2061	3/12/1999	9/12/1999	10/01/2000	78	30	35	y	44.87	48	72.92	27/03/2000	4/04/2000	16/05/2000	23	1	4.348	0.00	0.000	47.23
2062	3/12/1999	10/12/1999	10/01/2000	85	13	72		84.71	72	100.00	27/03/2000	13/04/2000	15/05/2000	67	1	1.493	0.00	0.000	47.36
2063	3/12/1999	10/12/1999	10/01/2000	107	65	42		39.25	42	100.00	27/03/2000	19/04/2000	20/06/2000	17	0	0.000	na	na	47.25
2064	3/12/1999	10/12/1999	10/01/2000	107	52	15		14.02	55	27.27	27/03/2000	26/04/2000	20/06/2000	6	0	0.000	na	na	47.47
2065	3/12/1999	10/12/1999	10/01/2000	86	27	51		59.30	59	86.44	27/03/2000	14/04/2000	15/05/2000	32	1	3.125	0.00	0.000	46.6
2066	3/12/1999	10/12/1999	10/01/2000	93	74	2		2.15	19	10.53	27/03/2000	3/05/2000	27/06/2000	1	0	0.000	na	na	46.71
			total seeds	1454	593	579		39.82	861	67.25				440	8	1.818	1	0.250	
			mean	90.875	37.063	36.188		39.964	53.813	62.906				27.500	0.500	1.253	0.200	0.050	
			std dev	13.140	17.487	22.660		24.302	13.881	34.901				25.553	1.033	2.027			

Table A 2.1.2 cont.

	Cass flats																		
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
2081	3/12/1999	5/12/1999	10/01/2000	108	72	0	y	0.00	36	0.0000	19/01/2000	X	X	0	0	na	na	na	na
2082	3/12/1999	5/12/1999	10/01/2000	102	64	0		0.00	38	0.0000	19/01/2000	X	X	0	0	na	na	na	na
2083	3/12/1999	5/12/1999	10/01/2000	120	97	1		0.83	23	4.3478	19/01/2000	27/01/2000	X	0	0	na	na	na	na
2084	3/12/1999	5/12/1999	10/01/2000	100	63	4		4.00	37	10.8108	1/02/2000	8/03/2000	11/04/2000	4	0	0.00	na	na	45.59
2085	3/12/1999	5/12/1999	10/01/2000	101	62	11		10.89	39	28.2051	1/02/2000	12/02/2000	29/03/2000	7	0	0.00	na	na	44.68
2086	3/12/1999	6/12/1999	10/01/2000	109	91	0		0.00	18	0.0000	1/02/2000	X	X	0	0	na	na	na	na
2087	3/12/1999	6/12/1999	10/01/2000	105	64	2		1.90	41	4.8780	9/03/2000	22/03/2000	9/05/2000	1	0	0.00	na	na	45.02
2088	3/12/1999	6/12/1999	10/01/2000	89	82	0		0.00	7	0.0000	9/03/2000	X	X	0	0	na	na	na	na
2089	3/12/1999	6/12/1999	10/01/2000	87	63	0		0.00	24	0.0000	9/03/2000	X	X	0	0	na	na	na	na
2090	3/12/1999	6/12/1999	10/01/2000	97	73	2		2.06	24	8.3333	9/03/2000	3/04/2000	9/05/2000	2	1	50.00	0.00	0.000	44.69
2091	3/12/1999	6/12/1999	10/01/2000	85	64	0	y	0.00	21	0.0000	9/03/2000	X	X	0	0	na	na	na	na
2092	3/12/1999	7/12/1999	10/01/2000	98	91	0		0.00	7	0.0000	9/03/2000	X	X	0	0	na	na	na	na
2093	3/12/1999	7/12/1999	10/01/2000	93	93	0		0.00	0	na	X	X	X	0	0	na	na	na	na
2094	3/12/1999	7/12/1999	10/01/2000	93	55	0		0.00	38	0.0000	27/03/2000	17/05/2000	X	0	0	na	na	na	na
2095	3/12/1999	7/12/1999	10/01/2000	98	95	0		0.00	3	0.0000	27/03/2000	X	X	0	0	na	na	na	na
2096	3/12/1999	10/12/1999	10/01/2000	103	102	0		0.00	1	0.0000	27/03/2000	X	X	0	0	na	na	na	na
2097	10/01/2000	13/01/2000	4/02/2000	86	34	52		60.47	52	100.0000	12/04/2000	19/05/2000	17/07/2000	42	0	0.00	na	na	45.02
2098	10/01/2000	13/01/2000	4/02/2000	60	39	13	y	21.67	21	61.9048	12/04/2000	28/04/2000	20/06/2000	10	0	0.00	na	na	45.34
2099	10/01/2000	13/01/2000	4/02/2000	64	39	19		29.69	25	76.0000	12/04/2000	19/05/2000	17/07/2000	12	0	0.00	na	na	45.7
2100	10/01/2000	13/01/2000	4/02/2000	84	50	23		27.38	34	67.6471	12/04/2000	19/05/2000	17/07/2000	19	0	0.00	na	na	44.98
2101	10/01/2000	13/01/2000	4/02/2000	33	28	1		3.03	5	20.0000	12/04/2000	26/04/2000	17/05/2000	1	0	0.00	na	na	na
2102	10/01/2000	14/01/2000	4/02/2000	78	24	50		64.10	54	92.5926	12/04/2000	19/05/2000	6/07/2000	5	0	0.00	na	na	45.4
2103	10/01/2000	15/01/2000	4/02/2000	63	50	7		11.11	13	53.8462	12/04/2000	19/05/2000	17/07/2000	5	0	0.00	na	na	45.54
2104	10/01/2000	16/01/2000	4/02/2000	96	22	72		75.00	74	97.2973	18/05/2000	23/06/2000	6/08/2000	8	0	0.00	na	na	45.72
2105	10/01/2000	16/01/2000	4/02/2000	88	20	58		65.91	68	85.2941	18/05/2000	23/06/2000	6/08/2000	39	0	0.00	na	na	45.76
			total seeds	2240	1537	315		14.06	703	44.8080				155	1	0.65	0	0.000	
			mean	89.600	61.480	12.600		15.122	28.120	29.632				6.200	0.040	3.846	0.000	0.000	
			std dev	18.664	25.272	21.446		24.490	20.080	37.735				11.391	0.200	13.868			

	Chilton valley																		
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
2111	3/12/1999	7/12/1999	10/01/2000	31	29	0	y	0.00	2	0.00	1/02/2000	X	X	0	0	na	na	na	na
2112	3/12/1999	7/12/1999	10/01/2000	103	102	0		0.00	1	0.00	22/02/2000	X	X	0	0	na	na	na	na
2113	3/12/1999	7/12/1999	10/01/2000	61	24	9		14.75	37	24.32	22/02/2000	17/03/2000	2/05/2000	9	0	0.00	na	na	47.11
2114	3/12/1999	10/12/1999	10/01/2000	89	80	0	y	0.00	9	0.00	22/02/2000	X	X	0	0	na	na	na	na
2115	3/12/1999	10/12/1999	10/01/2000	92	78	10		10.87	14	71.43	22/02/2000	20/03/2000	9/05/2000	10	0	0.00	na	na	na
2116	3/12/1999	10/12/1999	10/01/2000	99	6	50		50.51	93	53.76	9/03/2000	20/03/2000	2/05/2000	48	4	8.33	1.00	0.250	44.69
2117	3/12/1999	10/12/1999	10/01/2000	80	49	1		1.25	31	3.23	9/03/2000	10/04/2000	16/05/2000	0	0	na	na	na	na
2118	3/12/1999	10/12/1999	10/01/2000	133	64	10		7.52	69	14.49	9/03/2000	22/03/2000	X	0	0	na	na	0.000	na
2119	3/12/1999	10/12/1999	10/01/2000	106	96	0		0.00	10	0.00	19/01/2000	X	X	0	0	na	na	na	na
2120	3/12/1999	10/12/1999	10/01/2000	75	53	0		0.00	22	0.00	9/03/2000	17/05/2000	X	0	0	na	na	na	na
2121	10/01/2000	13/01/2000	4/02/2000	92	19	53		57.61	73	72.60	31/03/2000	3/05/2000	6/07/2000	38	1	2.63	0.00	0.000	36
2122	10/01/2000	13/01/2000	4/02/2000	62	58	2		3.23	4	50.00	31/03/2000	9/05/2000	6/07/2000	1	0	0.00	na	na	na
2123	10/01/2000	14/01/2000	4/02/2000	100	16	81		81.00	84	96.43	31/03/2000	2/05/2000	26/06/2000	51	0	0.00	na	na	36
2124	10/01/2000	14/01/2000	4/02/2000	89	15	55		61.80	74	74.32	31/03/2000	2/05/2000	27/06/2000	40	0	0.00	na	na	47.36
2125	10/01/2000	14/01/2000	4/02/2000	106	19	71		66.98	87	81.61	31/03/2000	13/04/2000	20/06/2000	52	0	0.00	na	na	36
2126	10/01/2000	15/01/2000	4/02/2000	100	12	63		63.00	88	71.59	31/03/2000	13/04/2000	18/05/2000	49	0	0.00	na	na	46.46
2127	10/01/2000	15/01/2000	4/02/2000	92	17	58		63.04	75	77.33	31/03/2000	3/05/2000	6/07/2000	42	1	2.38	0.00	0.000	46.17
2128	10/01/2000	15/01/2000	4/02/2000	99	73	19		19.19	26	73.08	31/03/2000	4/05/2000	27/06/2000	13	1	7.69	1.00	1.000	46.96
2129	10/01/2000	15/01/2000	4/02/2000	92	17	63		68.48	75	84.00	31/03/2000	28/04/2000	26/06/2000	38	0	0.00	na	na	45.56
2130	10/01/2000	16/01/2000	4/02/2000	106	16	78		73.58	90	86.67	31/03/2000	26/04/2000	20/06/2000	51	0	0.00	na	na	47.21
2131	10/01/2000	16/01/2000	4/02/2000	88	24	17		19.32	64	26.56	18/05/2000	23/06/2000	6/08/2000	18	0	0.00	na	na	36
2132	10/01/2000	16/01/2000	4/02/2000	85	33	5		5.88	52	9.62	18/05/2000	30/06/2000	10/08/2000	3	0	0.00	na	na	46.84
2133	10/01/2000	17/01/2000	4/02/2000	81	48	26		32.10	33	78.79	18/05/2000	27/06/2000	6/08/2000	9	0	0.00	na	na	46.89
2134	10/01/2000	17/01/2000	4/02/2000	113	20	18		15.93	93	19.35	18/05/2000	30/06/2000	10/08/2000	7	0	0.00	na	na	46.46
2135	10/01/2000	17/01/2000	4/02/2000	80	17	36		45.00	63	57.14	18/05/2000	28/06/2000	10/08/2000	18	0	0.00	na	na	46.58
2136	10/01/2000	17/01/2000	4/02/2000	104	67	4		3.85	37	10.81	18/05/2000	30/06/2000	10/08/2000	2	0	0.00	na	na	46.21
2137	10/01/2000	18/01/2000	4/02/2000	100	16	34		34.00	84	40.48	18/05/2000	30/06/2000	10/08/2000	23	0	0.00	na	na	46.62
2138	10/01/2000	18/01/2000	4/02/2000	79	20	43		54.43	59	72.88	18/05/2000	27/06/2000	10/08/2000	13	0	0.00	na	na	46.84
			total seeds	2537	1088	806		31.77	1449	55.62				535	7	1.31	2	1.250	
			mean	90.607	38.857	28.786		30.476	51.750	44.661				19.107	0.250	1.002	0.500	0.250	
			std dev	18.996	28.063	27.487		28.154	31.768	33.849				19.702	0.799	2.451			

Table A 2.1.2 cont.

	Little river																		
	Date sel	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
2141	27/01/2000	30/01/2000	27/02/2000	59	33	26		44.07	26	100.00	22/06/2000	27/07/2000	30/08/2000	22	0	0.00	na	na	44.71
2142	27/01/2000	2/02/2000	27/02/2000	87	46	41		47.13	41	100.00	22/06/2000	10/07/2000	16/08/2000	38	0	0.00	na	na	na
2143	27/01/2000	2/02/2000	27/02/2000	92	48	20		21.74	44	45.45	22/06/2000	27/07/2000	30/08/2000	12	0	0.00	na	na	45.32
2144	27/01/2000	2/02/2000	27/02/2000	74	14	45		60.81	60	75.00	22/06/2000	10/07/2000	24/08/2000	45	0	0.00	na	na	44.71
2145	27/01/2000	2/02/2000	27/02/2000	84	65	18		21.43	19	94.74	22/06/2000	17/07/2000	27/08/2000	11	0	0.00	na	na	44.98
2146	27/01/2000	6/02/2000	27/02/2000	91	16	58		63.74	75	77.33	22/06/2000	13/07/2000	25/08/2000	45	1	2.22	0.00	0.000	44.71
2147	27/01/2000	6/02/2000	27/02/2000	93	23	42		45.16	70	60.00	22/06/2000	19/07/2000	25/08/2000	27	0	0.00	na	na	36
2148	27/01/2000	6/02/2000	27/02/2000	83	46	32		38.55	37	86.49	22/06/2000	7/07/2000	16/08/2000	32	0	0.00	na	na	36
2149	27/01/2000	6/02/2000	7/03/2000	115	41	35		30.43	74	47.30	22/06/2000	27/07/2000	27/08/2000	16	0	0.00	na	na	45.43
2150	27/01/2000	6/02/2000	7/03/2000	77	31	32		41.56	46	69.57	22/06/2000	19/07/2000	30/08/2000	24	0	0.00	na	na	36
2151	27/01/2000	6/02/2000	7/03/2000	89	51	28		31.46	38	73.68	28/06/2000	29/07/2000	3/09/2000	18	0	0.00	na	na	45.68
2152	28/01/2000	6/02/2000	7/03/2000	75	43	11		14.67	32	34.38	28/06/2000	2/08/2000	3/09/2000	4	1	25.00	0.00	0.000	36
2153	27/01/2000	6/02/2000	7/03/2000	82	45	31		37.80	37	83.78	28/06/2000	31/07/2000	5/09/2000	24	2	8.33	0.00	0.000	45.74
2154	27/01/2000	6/02/2000	7/03/2000	58	23	35		60.34	35	100.00	28/06/2000	29/07/2000	30/08/2000	28	0	0.00	na	na	45.32
2155	27/01/2000	9/02/2000	7/03/2000	113	46	24		21.24	67	35.82	28/06/2000	6/08/2000	7/09/2000	12	0	0.00	na	na	45.32
2156	27/01/2000	9/02/2000	7/03/2000	97	37	32		32.99	60	53.33	28/06/2000	2/08/2000	3/09/2000	16	1	6.25	0.00	0.000	45.18
2157	27/01/2000	9/02/2000	7/03/2000	95	15	47		49.47	80	58.75	28/06/2000	29/07/2000	3/09/2000	26	0	0.00	na	na	na
2158	27/01/2000	9/02/2000	7/03/2000	93	58	21		22.58	35	60.00	28/06/2000	31/07/2000	3/09/2000	9	0	0.00	na	na	45.04
2159	27/01/2000	9/02/2000	7/03/2000	61	33	28		45.90	28	100.00	28/06/2000	29/07/2000	3/09/2000	23	1	4.35	0.00	0.000	44.69
2160	27/01/2000	9/02/2000	7/03/2000	75	32	35		46.67	43	81.40	28/06/2000	6/08/2000	3/09/2000	24	0	0.00	na	na	45.45
2161	9/02/2000	21/02/2000	7/03/2000	69	21	25		36.23	48	52.08	18/07/2000	8/08/2000	27/08/2000	21	2	9.52	1.00	0.500	44.84
2162	9/02/2000	21/02/2000	23/03/2000	76	24	25		32.89	52	48.08	18/07/2000	17/07/2000	7/09/2000	22	2	9.09	0.00	0.000	45
2163	9/02/2000	21/02/2000	23/03/2000	71	40	22		30.99	31	70.97	18/07/2000	17/07/2000	27/08/2000	19	2	10.53	0.00	0.000	44.8
2164	9/02/2000	24/02/2000	23/03/2000	72	35	36		50.00	37	97.30	18/07/2000	8/08/2000	8/09/2000	27	3	11.11	2.00	0.667	45.16
2165	9/02/2000	24/02/2000	23/03/2000	58	22	31		53.45	36	86.11	18/07/2000	16/08/2000	8/09/2000	20	1	5.00	0.00	0.000	45.34
2166	9/02/2000	24/02/2000	23/03/2000	97	66	20		20.62	31	64.52	18/07/2000	10/08/2000	8/09/2000	8	0	0.00	na	na	44.95
2167	9/02/2000	24/02/2000	23/03/2000	58	29	23		39.66	29	79.31	18/07/2000	16/08/2000	8/09/2000	17	0	0.00	na	na	44.35
2168	9/02/2000	24/02/2000	23/03/2000	80	49	22		27.50	31	70.97	18/07/2000	16/08/2000	8/09/2000	14	1	7.14	0.00	0.000	44.93
2169	9/02/2000	27/02/2000	23/03/2000	65	39	26		40.00	26	100.00	18/07/2000	15/08/2000	11/09/2000	20	0	0.00	na	na	44.8
2170	9/02/2000	27/02/2000	23/03/2000	57	33	13		22.81	24	54.17	18/07/2000	15/08/2000	11/09/2000	5	0	0.00	na	na	45.31
			total seeds	2396	1104	884		36.89	1292	68.42				629	17	2.70	3	1.167	
			mean	79.867	36.800	29.467		37.730	43.067	72.017				20.967	0.567	3.285	0.273	0.106	
			std dev	15.826	13.860	10.268		13.093	16.770	20.350				10.156	0.858	5.624			

Table A 2.1.3, 2000 / 2001 Field season: pollination dates and reproductive output

Cave Stream

	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
3001	15/12/2000	18/12/2000	4/01/2001	88	13	56		63.64	75	74.67	16/01/2001	21/03/2001	2/05/2001	16	0	0.00	na	na	47.3
3002	15/12/2000	18/12/2000	4/01/2001	76	16	8		10.53	60	13.33	16/01/2001	26/03/2001	2/05/2001	7	0	0.00	na	na	46.7
3003	15/12/2000	18/12/2000	4/01/2001	64	14	20		31.25	50	40.00	16/01/2001	21/03/2001	2/05/2001	8	0	0.00	na	na	47.0
3004	15/12/2000	18/12/2000	4/01/2001	76	65	1		1.32	11	9.09	6/03/2001	2/05/2001	2/05/2001	1	0	0.00	na	na	46.9
3005	15/12/2000	19/12/2000	4/01/2001	111	21	29		26.13	90	32.22	6/03/2001	29/05/2001	5/07/2001	20	0	0.00	na	na	46.9
3006	15/12/2000	19/12/2000	4/01/2001	86	61	2		2.33	25	8.00	6/03/2001	2/05/2001	2/05/2001	2	0	0.00	na	na	47.1
3007	15/12/2000	20/12/2000	4/01/2001	93	71	1		1.08	22	4.55	26/04/2001	14/06/2001	11/07/2001	1	0	0.00	na	na	46.2
3008	15/12/2000	20/12/2000	4/01/2001	82	79	0		0.00	3	0.00	26/04/2001	11/07/2001	X	0	0	na	na	na	na
3009	15/12/2000	20/12/2000	7/01/2001	79	10	60		75.95	69	86.96	26/04/2001	11/06/2001	16/07/2001	58	0	0.00	na	na	46.6
3010	15/12/2000	20/12/2000	7/01/2001	81	81	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3011	15/12/2000	21/12/2000	4/01/2001	53	53	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3012	15/12/2000	21/12/2000	4/01/2001	63	63	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3013	15/12/2000	21/12/2000	4/01/2001	73	73	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3014	15/12/2000	22/12/2000	7/01/2001	125	22	87		69.60	103	84.47	1/06/2001	17/07/2001	30/08/2001	84	2	2.38	1.00	0.500	46.6
3015	4/01/2000	8/01/2001	27/01/2001	39	17	11		28.21	22	50.00	1/06/2001	16/07/2001	20/08/2001	11	0	0.00	na	na	47.0
3016	4/01/2000	8/01/2001	27/01/2001	113	25	32		28.32	88	36.36	1/06/2001	30/07/2001	5/09/2001	32	1	3.13	0.00	0.000	47.0
3017	4/01/2000	9/01/2001	27/01/2001	97	10	33		34.02	87	37.93	1/06/2001	17/07/2001	22/08/2001	30	1	3.33	1.00	1.000	46.3
			total seeds	1399	694	340		24.30	705	48.23				270	4	1.48	2	1.500	
			mean	82.294	40.824	20.000		21.903	41.471	36.737				15.882	0.235	0.737	0.667	0.500	
			std dev	21.774	27.641	25.280		26.267	37.910	30.237				23.653	0.562	1.350		0.500	

NB// "infected refers to whether there was any fungal growth in the culture during germination.

***Dracophyllum* flat lower**

	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
3021	15/12/2000	18/12/2000	4/01/2001	80	66	0		0.00	14	0.00	16/01/2001	X	X	0	0	na	na	na	na
3022	15/12/2000	18/12/2000	4/01/2001	64	36	23		35.94	28	82.14	16/01/2001	8/03/2001	20/04/2001	14	14	100.00	8.00	0.571	35.9
3023	15/12/2000	18/12/2000	4/01/2001	98	98	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3024	15/12/2000	18/12/2000	4/01/2001	86	16	43		50.00	70	61.43	16/01/2001	21/03/2001	30/04/2001	25	0	0.00	na	na	46.5
3025	15/12/2000	19/12/2000	4/01/2001	57	0	57		100.00	57	100.00	6/03/2001	3/05/2001	X	0	0	na	na	na	na
3026	15/12/2000	19/12/2000	4/01/2001	60	38	0		0.00	22	0.00	6/03/2001	X	X	0	0	na	na	na	na
3027	15/12/2000	20/12/2000	4/01/2001	82	82	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3028	15/12/2000	20/12/2000	4/01/2001	79	79	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3029	15/12/2000	20/12/2000	4/01/2001	73	65	0		0.00	8	0.00	6/03/2001	X	X	0	0	na	na	na	na
3030	15/12/2000	20/12/2000	4/01/2001	76	76	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3031	15/12/2000	20/12/2000	4/01/2001	106	63	3		2.83	43	6.98	26/04/2001	21/06/2001	11/07/2001	3	0	0.00	na	na	44.9
3032	15/12/2000	20/12/2000	4/01/2001	76	76	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3033	15/12/2000	20/12/2000	4/01/2001	88	84	0		0.00	4	0.00	26/04/2001	11/07/2001	X	0	0	na	na	na	na
3034	15/12/2000	20/01/2001	7/01/2001	93	20	64		68.82	73	87.67	26/04/2001	11/06/2001	16/07/2001	61	6	9.84	1.00	0.167	47.0
3036	4/01/2001	7/01/2001	27/01/2001	76	39	2		2.63	37	5.41	1/06/2001	30/07/2001	5/09/2001	2	1	50.00	0.00	0.000	47.1
3037	4/01/2001	7/01/2001	27/01/2001	49	6	20		40.82	41	48.78	1/06/2001	20/07/2001	22/08/2001	20	20	100.00	9.00	0.450	40.2
3038	4/01/2001	7/01/2001	27/01/2001	65	48	17		26.15	17	100.00	1/06/2001	16/07/2001	22/08/2001	17	17	100.00	9.00	0.529	37.8
3039	4/01/2001	8/01/2001	27/01/2001	75	21	10		13.33	54	18.52	26/04/2001	21/06/2001	11/07/2001	10	0	0.00	na	na	47.2
			total seeds	1383	915	239		17.28	468	51.07				152	58	38.16	27	1.718	
			mean	76.833	50.833	13.278		18.918	26.000	39.302				8.444	3.222	44.980	5.400	0.344	
			std dev	14.525	29.645	20.795		29.334	25.389	41.761				15.455	6.576	48.420			

Table A 2.1.3 cont.

Dracophyllum flat upper

	Date set																			
	15/12/2000	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
3051	15/12/2000	18/12/2000	4/01/2001	65	2	63		96.92	63	100.00	16/01/2001	19/03/2001	20/04/2001	58	0	0.00	na	na	45.6
3052	15/12/2000	18/12/2000	4/01/2001	76	8	63		82.89	68	92.65	16/01/2001	2/04/2001	9/05/2001	41	0	0.00	na	na	46.7
3053	15/12/2000	19/12/2000	4/01/2001	75	11	57		76.00	64	89.06	16/01/2001	30/03/2001	9/05/2001	44	0	0.00	na	na	46.2
3054	15/12/2000	19/12/2000	7/01/2001	113	3	84		74.34	110	76.36	16/01/2001	2/04/2001	14/05/2001	46	0	0.00	na	na	45.7
3055	15/12/2000	20/12/2000	4/01/2001	69	6	63		91.30	63	100.00	6/03/2001	9/05/2001	11/06/2001	49	2	4.08	0.00	0.000	47.2
3056	15/12/2000	20/12/2000	4/01/2001	73	65	0		0.00	8	0.00	6/03/2001	X	X	0	0	na	na	na	na
3057	15/12/2000	20/12/2000	7/01/2001	90	11	49		54.44	79	62.03	6/03/2001	3/05/2001	31/05/2001	40	1	2.50	0.00	0.000	46.6
3058	15/12/2000	20/12/2000	7/01/2001	98	9	42		42.86	89	47.19	6/03/2001	29/05/2001	5/07/2001	27	1	3.70	0.00	0.000	46.9
3059	15/12/2000	20/12/2000	9/01/2001	96	13	42		43.75	83	50.60	26/04/2001	14/06/2001	11/07/2001	42	2	4.76	1.00	0.500	46.9
3060	15/12/2000	20/12/2000	7/01/2001	81	30	4		4.94	51	7.84	26/04/2001	21/06/2001	24/07/2001	4	0	0.00	na	na	47.5
3061	15/12/2000	21/12/2000	7/01/2001	81	81	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3062	15/12/2000	21/12/2000	7/01/2001	79	58	1		1.27	21	4.76	26/04/2001	14/06/2001	11/07/2001	1	0	0.00	na	na	47.1
3063	15/12/2000	21/12/2000	7/01/2001	79	8	58		73.42	71	81.69	1/06/2001	23/07/2001	5/09/2001	56	0	0.00	na	na	47.0
3064	15/12/2000	21/12/2000	7/01/2001	84	73	1		1.19	11	9.09	1/06/2001	30/07/2001	5/09/2001	1	0	0.00	na	na	46.9
3065	15/12/2000	22/12/2000	27/01/2001	86	2	84		97.67	64	100.00	1/06/2001	20/07/2001	3/09/2001	80	4	5.00	1.00	0.250	47.2
3066	4/01/2001	7/01/2001	27/01/2001	77	15	2		2.60	62	3.23	16/07/2001	5/09/2001	17/09/2001	3	0	0.00	na	na	45.4
3067	4/01/2001	7/01/2001	27/01/2001	80	3	77		96.25	77	100.00	16/07/2001	5/09/2001	2/10/2001	77	0	0.00	na	na	47.2
3068	4/01/2001	7/01/2001	27/01/2001	64	10	36		56.25	54	66.67	16/07/2001	3/09/2001	26/09/2001	36	0	0.00	na	na	45.8
3069	4/01/2001	8/01/2001	27/01/2001	88	5	52		59.09	83	62.65	16/07/2001	4/09/2001	2/10/2001	63	0	0.00	na	na	47.2
3070	4/01/2001	8/01/2001	27/01/2001	70	20	30		42.86	50	60.00	20/08/2001	26/09/2001	18/10/2001	30	0	0.00	na	na	45.7
3071	4/01/2001	8/01/2001	27/01/2001	50	27	0		0.00	23	0.00	20/08/2001	18/09/2001	X	0	0	na	na	na	na
3072	4/01/2001	8/01/2001	27/01/2001	69	6	37		53.62	63	58.73	20/08/2001	24/09/2001	17/10/2001	35	0	0.00	na	na	46.4
3073	4/01/2001	8/01/2001	27/01/2001	71	6	48		67.61	65	73.85	20/08/2001	26/09/2001	23/10/2001	47	2	4.26	0.00	0.000	45.6
3074	4/01/2001	9/01/2001	27/01/2001	72	3	63		87.50	69	91.30	20/08/2001	17/09/2001	12/10/2001	59	7	11.86	0.00	0.000	45.2
3075	4/01/2001	9/01/2001	27/01/2001	62	10	39		62.90	52	75.00	20/08/2001	17/09/2001	17/10/2001	47	0	0.00	na	na	46.5
			total seeds	1948	485	995		51.08	1463	68.01				886	19	2.14	2	0.750	
			mean	77.920	19.400	39.800		50.787	58.520	58.863				35.440	0.760	1.644	0.286	0.107	
			std dev	13.025	23.551	26.117		35.306	27.221	35.820				24.977	1.640	2.958			

Cass flats

	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
3081	15/12/2000	18/12/2000	4/01/2001	97	43	54		55.67	54	100.00	26/02/2001	11/04/2001	16/05/2001	22	0	0.00	na	na	45.7
3082	15/12/2000	18/12/2000	4/01/2001	90	30	60		66.67	60	100.00	26/02/2001	17/04/2001	21/05/2001	41	0	0.00	na	na	45.0
3083	15/12/2000	18/12/2000	4/01/2001	91	29	64		70.33	64	100.00	26/02/2001	19/04/2001	X	0	0	na	na	na	na
3084	15/12/2000	18/12/2000	4/01/2001	123	35	88		71.54	88	100.00	2/04/2001	29/05/2001	5/07/2001	8	0	0.00	na	na	45.8
3085	15/12/2000	18/12/2000	4/01/2001	83	20	61		73.49	63	96.83	2/04/2001	31/05/2001	28/06/2001	41	0	0.00	na	na	45.0
3086	15/12/2000	18/12/2000	4/01/2001	89	30	48		53.93	59	81.36	2/04/2001	31/05/2001	28/06/2001	37	4	10.81	2.00	0.500	44.1
3087	15/12/2000	18/12/2000	4/01/2001	100	37	63		63.00	63	100.00	2/04/2001	31/05/2001	4/07/2001	28	0	0.00	na	na	46.1
3088	15/12/2000	19/12/2000	4/01/2001	75	0	75		100.00	75	100.00	18/05/2001	6/07/2001	3/08/2001	75	1	1.33	0.00	0.000	45.0
3089	15/12/2000	19/12/2000	4/01/2001	81	10	71		87.65	71	100.00	18/05/2001	6/07/2001	10/08/2001	70	1	1.43	0.00	0.000	45.3
3090	15/12/2000	19/12/2000	7/01/2001	88	30	28		41.18	38	73.68	18/05/2001	6/07/2001	7/08/2001	27	1	3.70	1.00	1.000	44.8
3091	15/12/2000	19/12/2000	7/01/2001	85	31	54		63.53	54	100.00	3/07/2001	16/08/2001	17/09/2001	48	2	4.17	0.00	0.000	45.5
3092	15/12/2000	20/12/2000	4/01/2001	87	32	53		60.92	55	96.36	3/07/2001	10/08/2001	10/09/2001	52	2	3.85	1.00	0.500	45.3
3093	15/12/2000	20/12/2000	4/01/2001	74	18	56		75.68	56	100.00	3/07/2001	21/08/2001	17/09/2001	48	0	0.00	na	0.000	45.4
3094	15/12/2000	20/12/2000	7/01/2001	107	19	0	Y	0.00	88	0.00	20/08/2001	X	X	0	0	na	na	na	na
3095	15/12/2000	20/01/2001	7/01/2001	113	28	53		46.90	85	62.35	20/08/2001	24/09/2001	18/10/2001	51	2	3.92	1.00	0.500	45.5
3096	4/01/2001	8/01/2001	27/01/2001	76	16	38		50.00	60	63.33	20/08/2001	26/09/2001	23/10/2001	36	2	5.56	0.00	0.000	45.9
3097	4/01/2001	8/01/2001	27/01/2001	74	19	11		14.86	55	20.00	20/08/2001	28/09/2001	23/10/2001	11	0	0.00	na	na	46.0
3098	4/01/2001	8/01/2001	27/01/2001	80	22	23		28.75	58	39.86	5/09/2001	15/10/2001	13/11/2001	15	1	6.67	1.00	1.000	45.3
3099	4/01/2001	8/01/2001	27/01/2001	100	15	56		56.00	85	65.88	5/09/2001	15/10/2001	12/11/2001	66	1	1.52	1.00	1.000	45.5
			total seeds	1693	464	956		56.47	1229	77.79				676	17	2.51	7	4.500	
			mean	89.105	24.421	50.316		56.848	64.789	78.919				35.579	0.895	2.526	0.700	0.409	
			std dev	14.575	10.362	21.856		23.830	13.722	30.356				22.579	1.100	3.072			

Table A 2.1.3 cont.

	Chilton valley																			
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
3111	15/12/2000	19/12/2000	7/01/2001	74	9	55		74.32	65	84.62	26/02/2001	19/04/2001	28/05/2001	28	0	0.00	na	na	45.9
3112	15/12/2000	20/12/2000	7/01/2001	83	8	59		71.08	75	78.67	26/02/2001	19/04/2001	28/05/2001	22	0	0.00	na	na	47.5
3113	15/12/2000	20/12/2000	7/01/2001	99	28	71		71.72	71	100.00	26/02/2001	17/04/2001	28/05/2001	44	0	0.00	na	na	46.8
3114	15/12/2000	20/12/2000	7/01/2001	98	9	30		30.61	89	33.71	26/02/2001	24/04/2001	X	0	0	na	na	na	na
3115	15/12/2000	21/12/2000	7/01/2001	91	8	35		38.46	83	42.17	2/04/2001	31/05/2001	9/07/2001	30	0	0.00	na	na	47.5
3116	15/12/2000	21/12/2000	7/01/2001	102	7	75		73.53	95	78.95	2/04/2001	1/06/2001	19/07/2001	30	1	3.33	0.00	0.000	47.5
3117	15/12/2000	21/12/2000	7/01/2001	79	4	39		49.37	75	52.00	2/04/2001	1/06/2001	16/07/2001	34	0	0.00	na	na	47.2
3118	15/12/2000	21/12/2000	7/01/2001	79	6	65		82.28	73	89.04	18/05/2001	2/07/2001	24/07/2001	65	2	3.08	1.00	0.500	47.3
3119	15/12/2000	22/12/2000	7/01/2001	79	5	74		93.67	74	100.00	18/05/2001	10/07/2001	20/08/2001	72	2	2.78	0.00	0.000	47.3
3120	15/12/2000	22/12/2000	9/01/2001	106	28	69		65.09	78	88.46	18/05/2001	10/07/2001	30/08/2001	64	3	4.69	0.00	0.000	47.9
3121	15/12/2000	22/12/2000	27/01/2001	70	5	65		92.86	65	100.00	18/05/2001	2/07/2001	1/08/2001	51	1	1.96	0.00	0.000	47.8
3122	15/12/2000	22/12/2000	27/01/2001	64	34	5		7.81	30	16.67	3/07/2001	3/09/2001	25/09/2001	5	0	0.00	na	na	48.0
3123	4/01/2001	7/01/2001	27/01/2001	72	46	19		26.39	26	73.08	3/07/2001	10/08/2001	10/09/2001	18	0	0.00	na	na	47.4
3124	4/01/2001	7/01/2001	27/01/2001	94	17	65		69.15	77	84.42	3/07/2001	21/08/2001	17/09/2001	58	0	0.00	na	na	47.6
3125	4/01/2001	8/01/2001	27/01/2001	84	18	0		0.00	66	0.00	16/07/2001	5/09/2001	X	0	0	na	na	na	na
3126	4/01/2001	8/01/2001	27/01/2001	90	0	90		100.00	90	100.00	16/07/2001	4/09/2001	8/10/2001	85	0	0.00	na	na	48.1
3127	4/01/2001	8/01/2001	27/01/2001	102	3	99		97.06	99	100.00	16/07/2001	5/09/2001	8/10/2001	79	0	0.00	na	na	47.9
3128	4/01/2001	8/01/2001	27/01/2001	112	35	65		58.04	77	84.42	5/09/2001	4/10/2001	25/10/2001	53	0	0.00	na	na	48.2
3129	4/01/2001	8/01/2001	27/01/2001	82	7	75		91.46	75	100.00	5/09/2001	4/10/2001	31/10/2001	70	1	1.43	0.00	0.000	48.1
3130	4/01/2001	8/01/2001	27/01/2001	38	38	0		0.00	0	na	X	X	X	0	0	na	na	na	na
3132	4/01/2001	9/01/2001	27/01/2001	87	16	70		80.46	71	98.59	5/09/2001	5/10/2001	29/10/2001	69	1	1.45	0.00	0.000	48.2
3133	4/01/2001	9/01/2001	27/01/2001	61	40	6		9.84	21	28.57	5/09/2001	15/10/2001	X	0	0	na	na	na	na
3134	4/01/2001	9/01/2001	27/01/2001	92	64	19		20.65	28	67.86	5/09/2001	5/10/2001	29/10/2001	19	0	0.00	na	na	48.3
3135	4/01/2001	9/01/2001	27/01/2001	75	62	11		14.67	13	84.62	5/09/2001	4/10/2001	25/10/2001	11	0	0.00	na	na	48.3
3136	4/01/2001	9/01/2001	27/01/2001	106	20	79		74.53	86	91.86	5/09/2001	15/10/2001	9/11/2001	77	1	1.30	0.00	0.000	47.5
			total seeds	2119	517	1240		58.52	1602	77.40				984	12	1.22	1	0.500	
			mean	84.760	20.680	49.600		55.722	64.080	74.070				39.360	0.480	0.953	0.125	0.063	
			std dev	16.721	18.409	30.292		33.053	27.353	29.407				28.574	0.823	1.429	0.354	0.177	

	Little river																		
	Date set	Date poll	Date coll	no. seed	no. empty	no. germ	infected?	% total germ	no seed filled	% filled germ	date agar	date perlit	date pots	no. progeny	no. hybrids	% progeny hybrids	no. hybrids obl. sex	% hybrids obl. sex	DNA Ploidy
3141	16/01/2001	24/01/2001	20/02/2001	84	19	40		47.62	65	61.54	26/02/2001	11/04/2001	16/05/2001	8	0	0.00	na	na	45.2
3142	16/01/2001	24/01/2001	20/02/2001	64	30	21		32.81	34	61.76	26/02/2001	11/04/2001	14/05/2001	13	0	0.00	na	na	46.7
3143	16/01/2001	24/01/2001	20/02/2001	97	9	16		16.49	88	18.18	26/02/2001	24/04/2001	X	0	0	na	na	na	na
3144	16/01/2001	24/01/2001	20/02/2001	76	5	48		63.16	71	67.61	2/04/2001	29/05/2001	5/07/2001	9	0	0.00	na	na	46.7
3145	16/01/2001	26/01/2001	20/02/2001	66	13	53		80.30	53	100.00	2/04/2001	29/05/2001	26/06/2001	38	1	2.63	0.00	0.000	45.7
3147	16/01/2001	28/01/2001	20/02/2001	64	12	52		81.25	52	100.00	2/04/2001	29/05/2001	4/07/2001	18	0	0.00	na	na	45.9
3148	16/01/2001	28/01/2001	20/02/2001	61	4	57		93.44	57	100.00	18/05/2001	6/07/2001	8/08/2001	56	2	3.57	1.00	0.500	46.6
3149	16/01/2001	28/01/2001	20/02/2001	55	17	33		60.00	38	86.84	18/05/2001	5/07/2001	1/08/2001	32	0	0.00	na	na	46.8
3150	16/01/2001	28/01/2001	20/02/2001	53	23	9	y	16.98	30	30.00	18/05/2001	28/06/2001	24/07/2001	8	0	0.00	na	na	46.6
3152	16/01/2001	30/01/2001	20/02/2001	76	19	51		67.11	57	89.47	3/07/2001	22/08/2001	25/09/2001	51	3	5.88	1.00	0.333	46.8
3153	16/01/2001	30/01/2001	20/02/2001	58	21	37		63.79	37	100.00	3/07/2001	16/08/2001	17/09/2001	33	1	3.03	0.00	0.000	46.2
3154	16/01/2001	30/01/2001	20/02/2001	55	5	50		90.91	50	100.00	3/07/2001	3/08/2001	10/09/2001	49	3	6.12	0.00	0.000	46.1
3155	16/01/2001	30/01/2001	20/02/2001	61	12	34		55.74	49	69.39	3/07/2001	21/08/2001	17/09/2001	34	0	0.00	na	na	46.2
3156	24/01/2001	30/01/2001	20/02/2001	66	35	23		34.85	31	74.19	16/07/2001	3/09/2001	25/09/2001	22	0	0.00	na	na	46.6
3157	24/01/2001	1/02/2001	20/02/2001	56	4	52		92.86	52	100.00	16/07/2001	3/09/2001	26/09/2001	52	0	0.00	na	na	46.7
3158	24/01/2001	1/02/2001	5/03/2001	77	43	16		20.78	34	47.06	16/07/2001	4/09/2001	26/09/2001	17	0	0.00	na	na	46.6
3159	24/01/2001	1/02/2001	5/03/2001	76	52	8		10.53	24	33.33	5/09/2001	8/10/2001	31/10/2001	4	0	0.00	na	na	47.0
3160	26/01/2001	5/02/2001	5/03/2001	78	42	24		30.77	36	66.67	5/09/2001	8/10/2001	29/10/2001	21	0	0.00	na	ne	47.3
3161	26/01/2001	5/02/2001	5/03/2001	64	38	26		40.63	26	100.00	5/09/2001	12/10/2001	5/11/2001	24	0	0.00	na	na	46.6
3162	26/01/2001	5/02/2001	5/03/2001	63	15	44		69.84	48	91.67	5/09/2001	12/10/2001	31/10/2001	41	0	0.00	na	na	46.9
3163	1/02/2001	5/02/2001	5/03/2001	48	17	15		32.61	29	51.72	5/09/2001	12/10/2001	5/11/2001	14	0	0.00	na	na	46.2
			total seeds	1396	435	709		50.79	961	73.78				544	10	1.84	2	0.833	
			mean	66.476	20.714	33.762		52.498	45.762	73.783				25.905	0.476	1.062	0.400	0.167	
			std dev	12.023	14.125	16.106		26.702	16.321	26.264				17.099	0.981	2.031			

APPENDIX 3.1 TRIAL OF POLLINATION APPARATUS ON ABORTION RATE OF CAPITULA UNDER FIELD CONDITIONS.

To determine the effect of the pollination enclosures on seed set, twenty-five capitula were covered at the Cass flats, Chilton valley and Cave stream sites, and a further 25 over the two *Dracophyllum* flat sites. Capitula were harvested, and the seed recovered and examined. No germination trials of this seed were carried out, but the morphology was examined to see if seed were fully developed. The time between covering the inflorescences and recovery was eighteen days. The results can be seen in Table A3.1.1. As some research has suggested that the effects of enclosures can alter seed set and floral development (Bayer *et al.* 1990), this investigation was carried out to determine if this would be a considerable problem in studies of *Hieracium pilosella.*

Table A 3.1.1 Abortion frequency of capitula covered with pollination apparatus, seed collected after 18 days (% in parentheses).

Site	No. of Capitula covered	No. of Capitula w/- filled seed
Cass flats	25	20 (80.0%)
Chilton valley	25	25 (100.0%)
Dracophyllum flat lower	22	17 (77.3%)
Cave stream	25	25 (100.0%)

Although there was some abortion of capitula following the bagging of the inflorescence, this was not observed at all of the sites. The two sites where abortion did occur (Cass flats and *Dracophyllum* flat lower) had a higher incidence of abortion amongst uncovered capitula than the two remaining sites (G.J. Houliston *pers. obs.*). The lack of a measure of abortion rates in unbagged capitula prevents testing of whether bagging had a significant effect on abortion rates. That two sites also showed no increase in abortion rates following the bagging of capitula also indicates that this technique does not cause large scale detriment to the development of the inflorescence. Also not addressed was the number of seeds per capitula in bagged and unbagged treatments. It is possible that bagging had an effect on the fecundity of the plants. Due to the same method being used at each of the sites for the field study (see Chapter II.1, IV.1), this does not negatively effect the power of these experiments to determine relative rates or environmental effects.

APPENDIX 3.2 FREQUENCY OF CAPITULUM ABORTION FOLLOWING ARTIFICIAL POLLINATION UNDER FIELD CONDITIONS.

The following Tables (A3.2.1a-c) detail the number and frequency of capitula at each of the field sites over the three seasons of study that failed to produced any viable seed following artificial pollination.

Table A 3.2.1a Abortion Frequencies at the Field Sites (1998 / 1999).

Field Site	No. Capitula covered	No. Capitula with filled seed	No. Capitula aborted	% Capitula aborted
Cave stream	11	11	0	0.0%
Cass flats	18	18	0	0.0%
Chilton valley	24	22	2	8.3%
Dracophyllum flat lower	8	8	0	0.0%
Dracophyllum flat upper	8	8	0	0.0%
Little river	50	49	1	2.0%
Redcliffes station	NA	NA	NA	NA
Season totals	**119**	**116**	**3**	**2.52%**

Table A 3.2.1b Abortion Frequencies at the Field Sites (1999 / 2000).

Field Site	No. Capitula covered	No. Capitula with filled seed	No. Capitula aborted	% Capitula aborted
Cave stream	NA	NA	NA	NA
Cass flats	25	14	11	44.0%
Chilton valley	28	23	5	21.7%
Dracophyllum flat lower	13	2	11	84.6%
Dracophyllum flat upper	16	16	0	0.0%
Little river	30	30	0	0.0%
Redcliffes station	8	7	1	12.5%
Season totals	**120**	**92**	**28**	**23.33%**

Table A 3.2.1c Abortion Frequencies at the Field Sites (2000 / 2001).

Field Site	No. Capitula covered	No. Capitula with filled seed	No. Capitula aborted	% Capitula aborted
Cave stream	17	13	4	23.53%
Cass flats	19	19	0	0.00%
Chilton valley	25	24	1	4.00%
Dracophyllum flat lower	18	13	5	27.78%
Dracophyllum flat upper	25	24	1	4.00%
Little river	21	21	0	0.00%
Redcliffes station	NA	NA	NA	NA
Season totals	**125**	**114**	**11**	**8.8%**

APPENDIX 4 A SURVEY OF NEW ZEALAND POPULATIONS FOR OBLIGATE SEXUAL *HIERACIUM PILOSELLA.*

A4.1 Introduction

This reports the findings of a preliminary study into the potential for sexual reproduction in New Zealand populations of *Hieracium pilosella.* This study was carried out at the beginning of the research project to determine if obligate sexual *H. pilosella* was present in the New Zealand high country. Chapman *et al.* (2000) (as *Pilosella officinarum*) and Chapman and Brown (2001) found a high degree of morphological and genetic variation in *H. pilosella* populations, and this study aimed to determine if obligate sexual *H. pilosella* was responsible for the variation observed.

Populations of *Hieracium pilosella* collected from field sites throughout New Zealand (see Chapman *et al.* 2000 as *Pilosella officinarum*, Chapman & Brown 2001) were examined to determine if any obligate sexual individuals were present, over the spring of 1998.

The detection of obligate sexual individuals followed the method of Koltunow *et al.* (1995), removing the top 4 to 5mm of the capitula (see Figure 1.2.1) to ensure any seed set was the product of apomixis. Koltunow *et al.* (1995) states that the "degree of apomixis in *Hieracium pilosella* is easily assayed by decapitation of the unopened bud, which removes the stigma and anthers". The amount of seed produced via apomixis can then be directly counted. This method is not suitable to quantify the type of seed production in *H. pilosella*, although if no seed is filled it can indicate the presence of an obligate sexual plant. Methods for the quantification of the proportion of apomictic to sexually produced seed progeny require greater resolution than this method provides (see Chapter II). Physiological stress is known to cause the abortion of entire capitula, and is often implicated in the loss of some viable seed production (Jenkins 1992). Even under glasshouse conditions there is always a small proportion of the seed that remains unfilled, and this is not mitigated by the pollination of the capitulum. This makes it an unsuitable method for the determination of accurate ratios of sexual to asexually produced seed (although see Kashin & Chernyshova 1997). This experiment does not aim to determine

the levels of residual sexual reproduction in *H. pilosella,* only to determine whether any of the plants sampled from numerous different sites around New Zealand were obligate sexuals.

A4.2 Materials and Methods

The plants examined were from twenty different areas in the South Island, as well as one North Island site. The plants were grown in uniform conditions for the previous three years at 22°C, and in a 16 hour day-length to stimulate flowering for the period of this experiment. All plants were uniformly trimmed on the 6th of June 1998, and left to initiate flowering under artificial lighting.

The capitula were emasculated by removing the top 4mm of the bud, following the method of Koltunow *et al.* (1995). This effectively removes the anthers and stigma preventing any seed production via sexual pathways. Following the emasculation, inflorescences were left to develop until seed set. 538 capitula were emasculated from the 21 different populations, with varying sample sizes. The seed output of each capitulum was examined for failure to produce filled seed, and the proportion of filled and unfilled seed was estimated.

A4.3 Results

All of the capitula examined had a high proportion of filled seed, estimated to be at least 60% of the total seed produced. No inflorescences aborted, although a few failed to develop if emasculation was carried out too early in the development of the capitulum. Frequency of seed production varied temporally, with different peak times of seed production for the different populations.

A4.4 Discussion

This experiment confirmed the suggestion of earlier works, that obligate sexual plants of *Hieracium pilosella* are not common, if at all present, in New Zealand populations (Makepeace 1981, Jenkins 1992). This would indicate, in respect to the high levels of genotypic variation present in New Zealand populations of *H. pilosella*, that it is facultative apomicts that are responsible for the occurrence of sexual events. This is at odds with the patterns found in Europe, where obligate sexual plants are documented as being common-place (Gadella 1972, 1987, 1991a, 1991b, Stace 1997) and also a foci for the generation of new types (Schuhwerk & Lippert 1991), particularly in crosses with apomicts as the pollen parent (Krahulcová *et al.* 2000).

More recent discoveries of obligate sexual *Hieracium pilosella* from the Canterbury region (see Chapter II.1) are interesting in respect to the findings of this investigation. A relatively large number of populations included in this study yielded no obligate sexual plants, yet out of the seven populations included in chapter II.1 two were found to contain obligate sexual individuals. The explanations for this are numerous, although perhaps one of the most parsimonious is relatively simple. Observations of the obligate sexual plants from both *Dracophyllum* flat lower and Redcliffes station growing under glasshouse conditions demonstrated that they had much lower vigour and higher mortality than apomictic lines. If obligate sexual plants were originally collected and had once been present in the glasshouse populations, their absence as found in the present study could be explained by complete mortality, or displacement by apomicts, of all sexual plants prior to this survey. Conversely, the selection of sites for this study may simply have not overlapped with areas with a high occurrence of sexual biotypes. The possibility for at least some residual sexual reproduction in all of the populations examined in detail (Chapter II.1), and the segregation of apomixis and sex in crosses, indicates that it is unlikely that obligate sexual individuals have not arisen elsewhere in New Zealand. However, due to the high degree of population variation detected in populations with facultative apomicts only, it is clear that facultative sex is possibly still the most important mechanism for the generation of variation in this species in New Zealand.

APPENDIX 5 CLIMATE DATA.

Table A 5.1.1 Mean values of environmental parameters for the week preceding pollination – Cass flats

Date of pollination	Rainfall	Temperature	Relative Humidity
08/01/99	0.000	19.35	65.53
09/01/99	0.000	19.19	62.29
11/01/99	0.000	18.50	62.27
05/12/99	0.040	14.78	64.55
06/12/99	0.033	15.19	63.76
07/12/99	2.857	14.65	65.53
10/12/99	2.829	13.91	68.88
13/01/00	0.086	13.01	78.84
14/01/00	0.086	12.09	78.83
15/01/00	0.086	12.73	80.00
16/01/00	0.000	13.49	79.04
18/12/00	0.371	17.65	67.12
19/12/00	0.286	18.08	63.98
20/12/00	0.286	18.41	61.24
08/01/01	0.943	14.49	72.04

Table A 5.1.2 Mean values of environmental parameters for the week preceding pollination – Chilton valley

Date of pollination	Rainfall	Temperature	Relative Humidity
08/01/99	0.000	18.35	58.80
10/01/99	0.000	17.75	59.52
11/01/99	0.000	17.29	60.32
07/12/99	2.543	12.96	67.31
10/12/99	2.514	12.28	69.93
13/01/99	0.200	11.65	78.77
14/01/99	0.200	11.67	77.59
15/01/99	0.200	11.73	78.58
16/01/99	0.000	12.50	77.60
17/01/99	0.000	12.88	75.76
18/01/99	0.000	13.52	72.57
19/12/00	0.136	16.08	65.48
20/12/00	0.136	16.41	62.74
21/12/00	0.136	15.96	63.34
07/01/01	1.821	11.06	73.34
08/01/01	0.793	12.49	73.54
09/01/01	0.793	13.65	72.84

Table A 5.1.3. Mean values of environmental parameters for the week preceding pollination – Little river

Date of pollination	Rainfall	Temperature	Relative Humidity
19/01/99	0.629	16.83	85.84
25/01/99	0.171	15.58	78.10
02/02/99	0.943	15.33	78.66
03/02/99	0.943	14.92	78.66
19/02/99	0.000	19.79	66.67
30/01/00	1.000	15.33	74.31
02/02/00	1.425	13.19	75.38
06/02/00	1.325	14.89	72.13
09/02/00	0.000	15.58	72.51
21/02/00	2.150	13.93	92.69
24/02/00	1.475	14.84	82.76
27/02/00	0.400	15.81	75.21
24/01/01	0.286	12.77	74.76
26/01/01	1.229	12.90	73.20
28/01/01	1.200	12.30	71.19
30/01/01	1.200	12.11	77.19
01/02/01	2.343	12.01	75.99
05/02/01	1.686	15.27	68.56

Table A 5.1.4 Climate data, Field Sites Summer 1998-2001. Recorded at Cass Field Station, Cass flats, Department of Plant & Microbial Sciences, U. of C.

	Rainfall (mm)	Air Temperature (°C)	Relative humidity (%)	Average Solar Radiation Li 200 (W/m2)	Minimum Air Temperature (°C)	Maximum Air Temperature (°C)
Dec 1998	77.0	13.7	72.5	278.4	8.0	21.2
Jan 1999	14.4	17.5	67.2	290.3	9.1	25.8
Feb 1999	34.8	17.2	63.8	263.7	8.1	26.0
Dec 1999	57.6	12.9	70.9	290.6	6.6	19.7
Jan 2000	201.6	14.1	77.6	248.8	8.0	21.0
Feb 2000	36.0	15.4	70.7	233.5	9.3	21.8
Dec 2000	70.0	15.3	63.6	306.9	8.1	22.3
Jan 2001	38.2	13.9	66.9	305.2	6.6	21.3
Feb 2001	13.8	15.4	66.2	248.2	8.3	24.1

Table A 5.1.5 Climate data, Field Sites Summer 1998-2001.
Recorded at Chilton Weather Station, Chilton valley, Department of Geography, U. of C.

	Rainfall (mm)	Air Temperature (°C)	Relative humidity (%)	Average Solar Radiation Li 200 (W/m2)	Minimum Air Temperature (°C)	Maximum Air Temperature (°C)
Dec 1998	74.6	12.3	73.0	246.7	7.519	18.3
Jan 1999	10.0	16.1	66.9	283.2	9.905	23.3
Feb 1999	109.2	15.5	65.7	231.2	9.581	22.2
Dec 1999	53.6	11.1	72.1	274.5	5.930	17.4
Jan 2000	195.0	12.7	78.2	236.5	7.759	18.6
Feb 2000	37.0	13.8	71.3	217.3	8.637	20.1
Dec 2000	NA	NA	NA	NA	NA	NA
Jan 2001	NA	NA	NA	NA	NA	NA
Feb 2001*	1.0	15.9	68.4	198.3	10.893	22.6

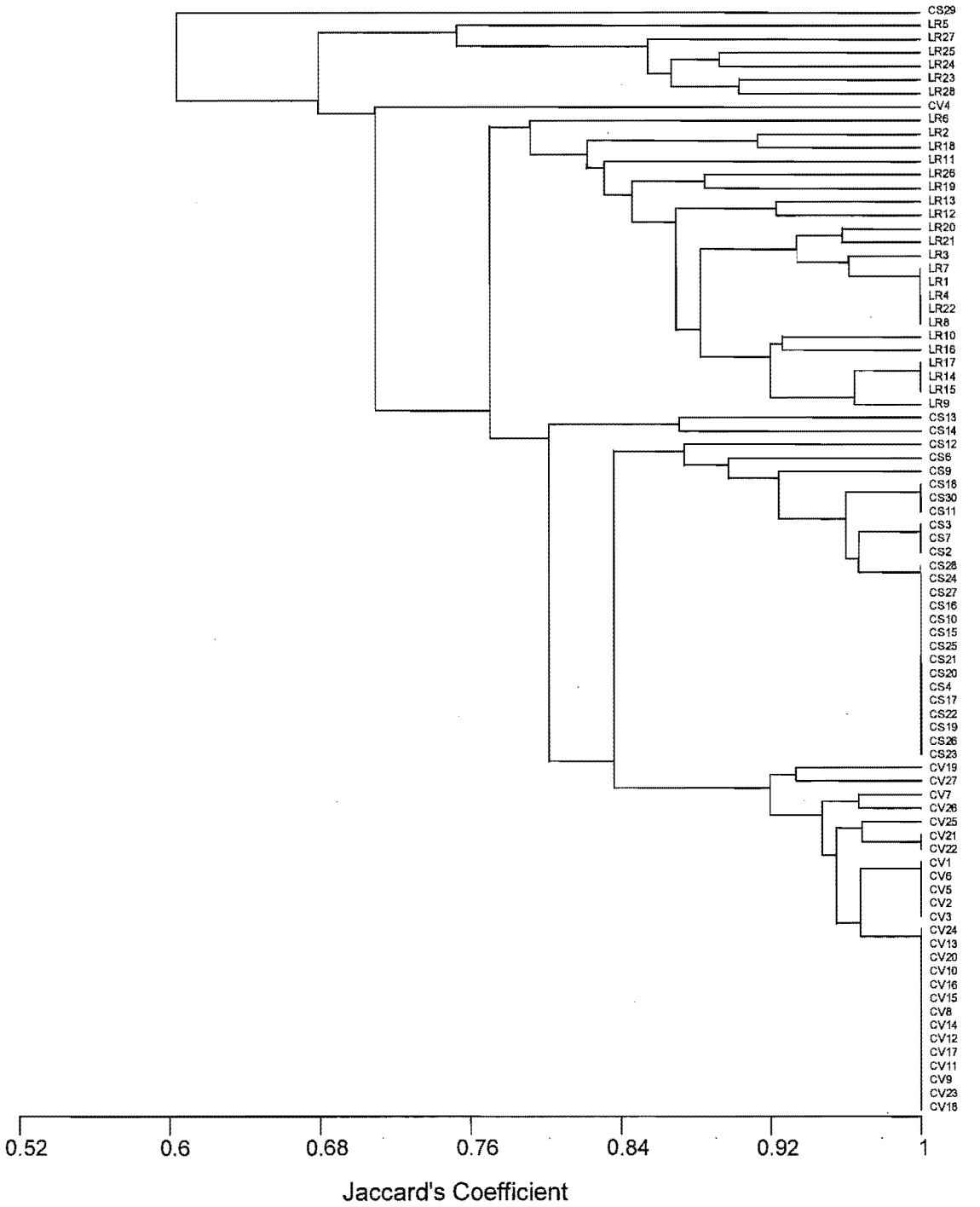
*Data from 14th of February onwards only

Table A 5.1.6 Climate data, Field Sites Summer 1998-2001.
Recorded at Banks Peninsula, Le Bons Bay, 236m asl, N.I.W.A. (Anonymous 1998, 1999a, 1999b, 1999c, 2000a, 2000b, 2000c, 2001a, 2001b).

	Air Temperature (°C)	Rainfall Total (mm)	Minimum Air Temperature (°C)	Maximum Air Temperature (°C)
Dec 1998	18.2	NA	11.2	19.0
Jan 1999	16.7	18	13.0	20.3
Feb 1999	16.6	NA	13.2	19.9
Dec 1999	12.8	46	9.4	16.1
Jan 2000	14.1	48	11.2	17.0
Feb 2000	15.2	18	11.2	19.2
Dec 2000	14.9	53	NA	NA
Jan 2001	NA	NA	NA	NA
Feb 2001	15.6	21	6.9	28.6

APPENDIX 6 DENDROGRAM OF ALL INDIVIDUALS INCLUDED IN THE POPULATION STUDY

Figure A 6.1 UPGMA dendrogram, all sites combined, ISSR data, Jaccards clustering.



APPENDIX 7 PARTIAL RESIDUAL PLOTS FOR VARIATES; CLIMATE AND ALL SITE MODELS.

Appendix 7.1.1 Climate sites – Partial Residual Plots

Figure A 7.1.1a Partial Residual Plot of Rainfall, Climate sites analysis

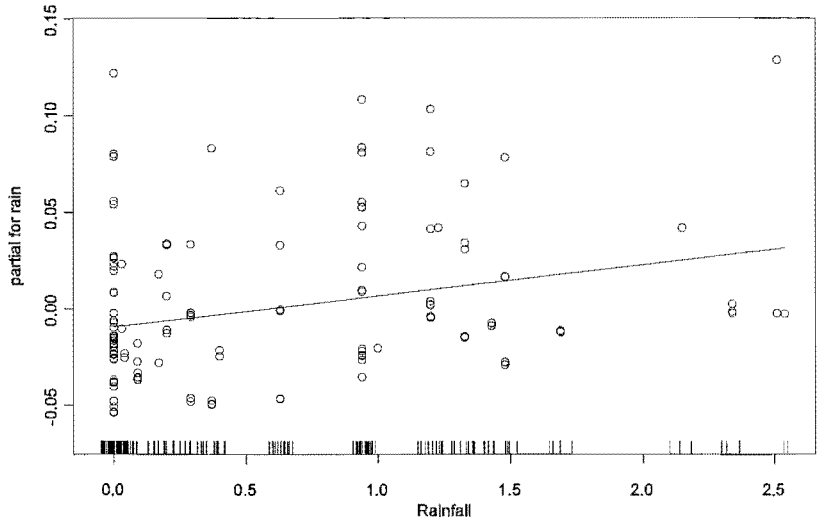


Figure A 7.1.1b Partial Residual Plot of Temperature, Climate sites analysis

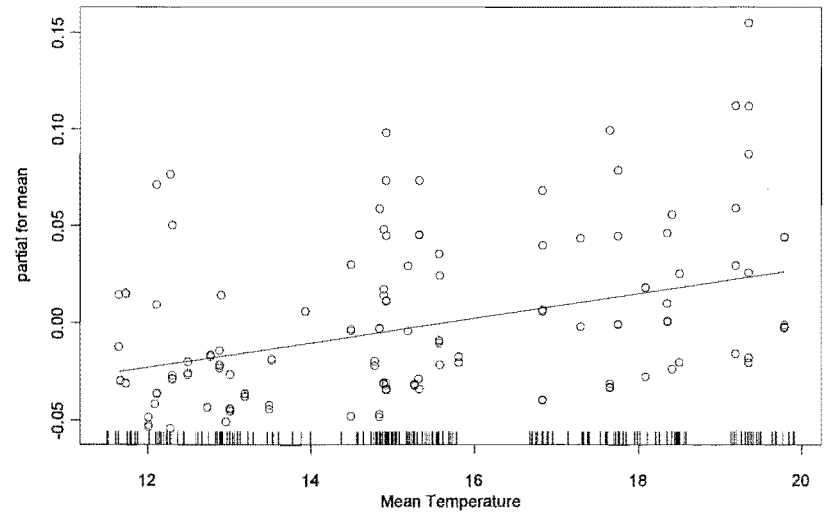
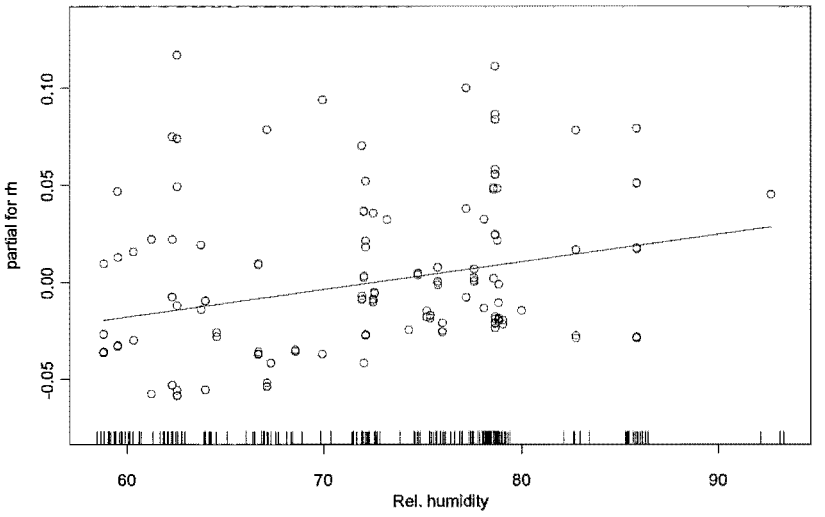


Figure A 7.1.1c Partial Residual Plot of Relative Humidity, Climate sites analysis



Appendix 7.1.2

Table A 7.1.2 Correlation of Predictors, Climate Site Analysis

Correlation of Coefficients:

	(Intercept)	Altitude 1	Altitude 2	Rel. humidity	Temperature
Altitude 1	0.40949				
Altitude 2	-0.51142	-0.17483			
Rel. humidity	-0.91587	-0.46474	0.44603		
Temperature	-0.86109	-0.22790	0.47267	0.59144	
Rainfall	-0.33281	-0.42411	0.36338	0.13429	0.43462

Appendix 7.2.1 All sites – Partial Residual Plots

Figure A 7.2.1a Partial Residual Plot of Altitude, All sites analysis

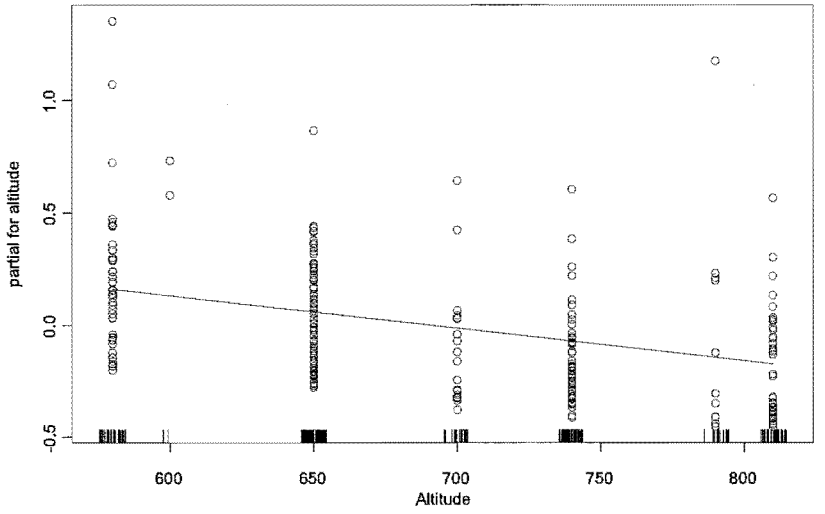


Figure A 7.2.1b Partial Residual Plot of Rank moisture, All sites analysis

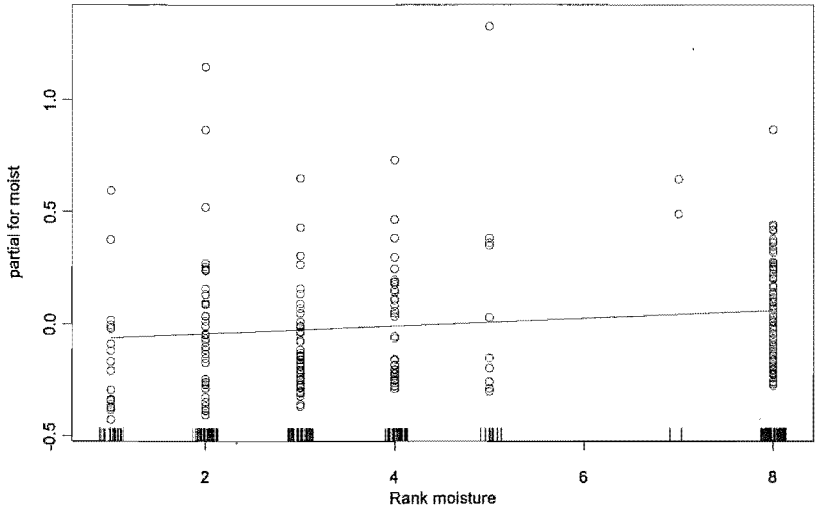
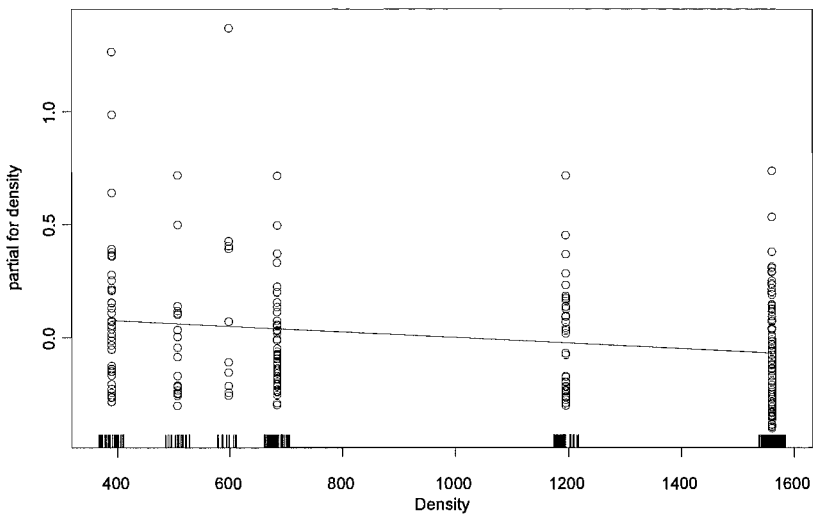


Figure A 7.2.1c Partial Residual Plot of Density, All sites analysis



Appendix 7.2.2

Table A 7.2.2 Correlation of predictors, All sites analysis

Correlation of Coefficients:

	(Intercept)	Altitude	Rank moisture
Altitude	-0.97511		
Rank moisture	-0.48388	0.54203	
Density	0.42415	-0.53434	-0.95943

APPENDIX 8 SEED PRODUCTION, COMMON GARDEN EXPERIMENT – GRAPHS OF SEED VIABILITY.

Figure A 8.1.1 Seed production under glasshouse conditions, Cass flats, Chilton valley & *Dracophyllum* flat lower sites.